REMARKS

Applicants thank the Examiner for the very helpful telephonic interview of July 19, 2006, in which proposed claim amendments were discussed. Further to that interview, applicants have amended claims 1 and 7-9 to remove the alkoxyl group from position R_3. Applicants have also amended claim 10 to incorporate each and every element of dependent claim 15. Claims 15 and 18-29 have been cancelled.

Applicants reserve the right to pursue any cancelled subject matter in this or a continuing application. No new matter has been added by any of these amendments.

With regard to the cancers listed in amended claim 10, applicants note that each one of these cancers is known to be associated with a protein containing a Bcl-2-homology-3 (BH3) domain. Support for this association may be found in Thomadaki and Scorlias, *Crit. Rev. Clin. Lab. Sci.* 43:1-67, 2006, enclosed as "Exhibit A." In particular, applicants point to Table 6 on page 36 of Exhibit A. This table lists associations between the recited cancers and several genes coding for proteins, each of which contains a BH3 domain (see Figure 5 on page 6). Support for the involvement of a BH3 domain-containing protein and cancer may also be found at page 42, lines 30-37, where it is noted that BFL1/A1 is involved with melanoma. Further support for treating cancer by the disruption of interaction between a BH3-containing protein and another protein may be found in Roa et al., *Clin. Invest. Med.* 28:55-63, 2005, enclosed as "Exhibit B." This

reference indicates that a BH3 small molecule mimetic that disrupts BH3 protein-protein interactions enhances radiation sensitivity of cancer cells, as shown in Figure 4.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Jeffrey C Kessler for

Jeffrey C. Kessler
Reg. No. 47,127

Date: 19 July 2006

Kristina Bieker-Brady, Ph.D.
Reg. No. 39,109

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

EXHIBIT A

Critical Reviews in Clinical Laboratory Sciences, 43(1):1–67 (2006)

ISSN: 1040-8363 print / 1549-781X online
DOI: 10.1080/10408360500295626

Taylor & Francis
Taylor & Francis Group

BCL2 FAMILY OF APOPTOSIS-RELATED GENES: Functions and Clinical Implications in Cancer

Hellinida Thomadaki □ *Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, Athens, Greece*

Andreas Scorilas □ *Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, Athens, Greece*

Referee Dr. J. Thomas Hindmarsh, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, Canada

□ *One of the most effective ways to combat different types of cancer is through early diagnosis and administration of effective treatment, followed by efficient monitoring that will allow physicians to detect relapsing disease and treat it at the earliest possible time. Apoptosis, a normal physiological form of cell death, is critically involved in the regulation of cellular homeostasis. Dysregulation of programmed cell death mechanisms plays an important role in the pathogenesis and progression of cancer as well as in the responses of tumours to therapeutic interventions.*

Many members of the BCL2 *(B-cell CLL/lymphoma 2; Bcl-2) family of apoptosis-related genes have been found to be differentially expressed in various malignancies, and some are useful prognostic cancer biomarkers. We have recently cloned a new member of this family,* BCL2L12, *which was found to be differentially expressed in many tumours. Most of the* BCL2 *family genes have been found to play a central regulatory role in apoptosis induction. Results have made it clear that a number of coordinating alterations in the* BCL2 *family of genes must occur to inhibit apoptosis and provoke carcinogenesis in a wide variety of cancers. However, more research is required to increase our understanding of the extent to which and the mechanisms by which they are involved in cancer development, providing the basis for earlier and more accurate cancer diagnosis, prognosis and therapeutic intervention that targets the apoptosis pathways.*

In the present review, we describe current knowledge of the function and molecular characteristics of a series of classic but also newly discovered genes of the BCL2 *family as well as their implications in cancer development, prognosis and treatment.*

Keywords Apoptosis, *BCL2* gene family, *BCL-2*, *BCL2L12*, cancer, cancer prognosis, cancer treatment, tumour biomarkers, *BCL2L1*, *MCL1*, *BCL2L2*, *BOO/DIVA*, *BCL2A1*, *BAX*, *BAK1*, *BOK*, *BIK*, *BID*, *HRK*, *BCL2L11*, *BNIP*, *BNIP1*, *BNIP2*, *BNIP3*, *NIX*, *cdBNIP3*, *BLK*, *PMAIP1*, *BMF*, *BBC3*, *BCLAF1*, *BCL2L13*, *BCL2L14*, *MAP1*, *BCL B*, *BCL2L10*, *BFK*, AIDS, Alzheimer, Parkinson, spinal muscular atrophy, Hashimoto thyroiditis, glomerulonephritis, Emery-Dreifuss muscular dystrophy.

Address correspondence to Dr. Andreas Scorilas, Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, Panepistimiopolis, 15701 Athens, Greece. E-mail: ascorilas@biol.uoa.gr or scorilas@netscape.net

Abbreviations and Glossary **A549**, lung cancer cells; **AD**, Alzheimer's disease; **AIDS**, acquired immunodeficiency syndrome, **Akt**, protein kinase B; **ALL**, acute lymphocytic leukemia, **AML**, acute myeloid leukemia; **ANT**, adenine nucleotide transporter; **Apaf1**, apoptosis-activating factor 1; **ATLL**, acute T lymphocytic leukemia; **BAD**, BCL2-antagonist of cell death; **BAF-3**, human bone marrow-derived cells; **BAK1**, BCL2-antagonist/killer 1; **BAX**, BCL2-associated X protein; **BBC3**, BCL2 binding component 3; **B-CLL**, B-cell chronic lymphocytic leukemia; **BCL2**, B-cell CLL/lymphoma 2; **BCL2L1**, BCL2 like protein 1; **BCL2L10**, BCL2-like protein 10; **BCL-Xγ**, BCL-X gamma form; **BCL-X$_L$**, BCL-X long form; **BCL-X$_S$**, BCL X short form; **BCR/ABL**, breakpoint cluster region/Abelson leukemia; **BFL1$_S$**, BFL short form; **BH**, BCL2 homology region; **BHRF-1**, Epstein-Barr virus BCL2 homologue; **BID**, BH3-interacting domain agonist; **BIK**, BCL2-interacting killer; **BIM**, BCL2-interacting mediator; **BMF**, BCL2-modifying factor; **BNIP**, BCL2 and the nineteen kDa interacting protein; **BOK**, BCL2-related ovarian killer; **BOK$_S$**, BOK short form; **BOO**, BCL2 homologue of the ovary; **BTF**, BCL2-associated transcription factor; **BTF$_L$**, BTF long form; **BTF$_S$**, BTF short form; **CD**, conserved domain; **CD40**, cluster of differentiation 40; **CD40L**, CD40 ligand; **ceBNIP3**, C. elegans BNIP3; **CED**, cell death protein; **CGH**, comparative genomic hybridization; **CMML**, chronic myelomonocytic leukemia; **CPP32**, cysteine protease protein of molecular mass 32 kDa; **CPT**, camptothecin, **CVB3**, coxsackievirus B3; **CWR22**, human prostate cancer xenograft; **DA**, dopaminergic neurons; **DCM**, dilated cardiomyopathy; **DIVA**, death inducer binding to vBCL2 and apoptosis-activating factor, APAF-1; **DLC**, dynein light chain; **DREAM**, downstream regulatory element antagonist modulator; **DS**, Down syndrome; **DT40**, bursal lymphoma cell line; **EDMD**, Emery Dreifuss muscular dystrophy; **EGF**, epidermal growth factor; **ER**, endoplasmic reticulum; **ERK**, extracellular signal-regulated kinase; **FasL**, Fas-ligand; **FDC-P1**, mouse promyelocytic cells; **FSH**, follicle-stimulating hormone; **GAP**, GTPase-activating protein; **GBM**, glioblastoma multiform; **G-CSF**, granulocyte colony-stimulating factor; **GM-CSF**, granulocyte macrophage colony-stimulating factor; **GTN**, gestational trophoblastic neoplasia; **HCC-T**, hepatocellular carcinoma; **HeLa**, human cervix carcinoma epithelial cells; **HL-CR**, ceramide resistant HL-60 subline; **HM**, hydatidiform mole; **HNSCC**, head/neck squamous cell carcinoma; **HPV**, human papillovirus; **HRK**, Harakiri; **HTLV**, human T lymphotropic virus; **IAPs**, inhibitors of apoptosis; **IGF-1**, insulin-like growth factor-1; **JAK**, Janus kinase; **JNK**, c-Jun NH2-terminal kinase; **IFN-α**, interferon-alpha; **IL**, interleukin; **IRF3**, interferon regulatory factor 3; **JHU**, head/neck cancer cells; **K562/AC**, K562 cell line Ara-C resistant; **KLK**, kallikrein; **L929**, mouse fibroblasts; **LNCaP**, prostatic carcinoma cell line; **LOH**, loss of heterozygosity; **LPS**, lipopolysaccharide; **MA**, membrane anchor; **MAP**, mitogen-activated protein; **MAP-1**, modulator of apoptosis-1; **MAPK1**, MAP kinase 1; **MCL1**, myeloid cell leukemia 1; **MCL1$_L$**, MCL1 long form; **MCL1$_S$**, MCL1 short form; **MCF-7**, human epithelial breast cancer cells; **MEK**, MAPK/ERK kinase; **Met**, a proto-oncogene encoding the tyrosine kinase growth factor receptor for hepatocyte growth factor; **ML-1**, human myeloblastic leukaemia cells; **MM**, maligoant melanoma; **MMP**, mitochondrial membrane potential; **NF-κB**, nuclear factor kappa B; **NGF**, nerve growth factor; **NLS**, nuclear localization signal; **NOD**, non-obese diabetic; **NOXA**, a proapoptotic BH3-only member of the BCL2 protein family; **PAK**, p21-activated kinase, **PEST**, sequence enriched in proline/glutamic acid/serine/threonine; **PDGF**, platelet-derived growth factor; **PI3K**, phosphatidylinositol 3-kinase; **PKA**, protein kinase A; **PLFZ**, promyelocytic leukaemia zinc-finger gene; **PMA**, phorbol ester; **PT**, permeability transition; **PTP**, permeability transition pore; **PUMA**, p53 up-regulated modulator of apoptosis; **Raf**, oncogene; **Ras**, oncogene; **ROS**, reactive oxygen species; **RRAS**, Ras-related protein R-Ras; **RSK**, ribosomal S6 kinase; **RT-PCR**, reverse transcription-polymerase chain reaction; **Saos2**, human osteosarcoma cells; **SH3**, src homology 3; **SH-SY5Y**, human neuroblastoma cells; **SLE**, systemic lupus erythematosus; **SRF**, serum factor; **SSCP**, single-strand conformation polymorphism;

STAT3, signal transducers and activators of transcription; **tBAD**, truncated BAD; **tBID**, truncated BID; **TCC**, transitional cell carcinoma; **TF1**, myeloid progenitor cell line; **TM**, transmembrane; **TNF**, tumour necrosis factor; **TRAIL**, tumour necrosis factor-related apoptosis-inducing ligand; **Ubl-L**, ubiquitin-like; **U87**, human glioblastoma cells; **U251**, human glioblastoma cells; **U138**, human glioblastoma cells; **VDAC**, voltage-dependent anion channels; **VEGF**, vascular endothelial growth factor; **WT**, wild type.

I. INTRODUCTION

Apoptosis is a genetically regulated form of cell death that occurs when the cell is exposed to physiological, pathogenic or cytotoxic stimuli. The main physical and biochemical hallmarks of apoptosis include loss of sialic acid, translocation of phosphatidylserine to the outer plasma membrane, cell shrinkage, nuclear condensation, chromatin aggregation, DNA endonucleolytic degradation, membrane blebbing and formation of apoptotic bodies, which are subsequently engulfed by neighbouring cells or phagocytes, preventing an inflammatory reaction.[1] This type of programmed cell death exists in all multicellular organisms and plays an indispensable and integral role in a variety of physiological procedures, such as embryonic development and morphogenesis, by diminishing unwanted or excessive cells (Figure 1). Furthermore, cell death helps the organism to maintain its homeostasis, being opposed to cell division (Figure 2). The cells that have been damaged by aging or by exposure to DNA-damaging agents or viruses, as well as autoimmune cells, are also eliminated by apoptosis.[2]

Aberrant regulation of apoptosis (too much or too little apoptosis) (Figure 2), at any time from embryogenesis to adulthood, can result in a variety of disease states, such as Acquired Immunodeficiency Syndrome (AIDS), neurodegenerative disorders, autoimmunity and cancer. A reasonable estimation is that either too little or too much cell death contributes to half of the main medical illnesses for which adequate therapy or prevention is lacking.

When the homeostatic balance is disturbed in such a way that clonal outgrowth of mutated cell populations may occur, this results in the development of a tumour.[3,4] Early in transformation, activated oncogenes that

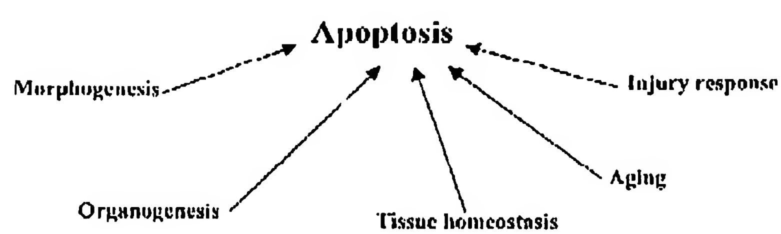

FIGURE 1 Physiological role of apoptosis.

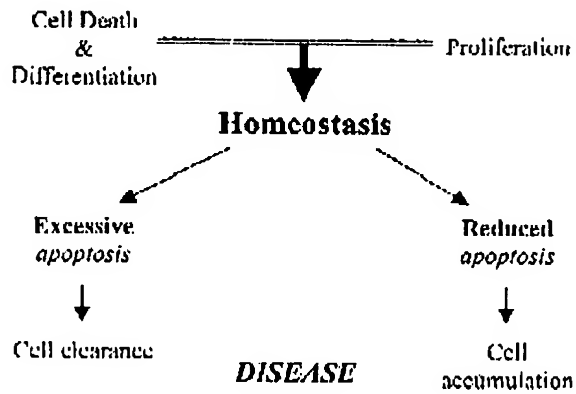

FIGURE 2 Aberrant regulation of apoptosis.

drive the cell into uncontrolled proliferation simultaneously trigger the cell-death programme, probably as a safety mechanism that removes cells carrying mutations in oncogenes.[5] Later in tumorigenesis, the supply of nutrients and oxygen becomes limited, with the tumour cells undergoing hypoxia induced apoptosis.[6] In order for tumour cells to survive, they acquire apoptotic-inhibiting mutations.[6] This is the main reason by which activation of apoptosis is one of the most potent therapeutic approaches in cancer treatment.

Apoptosis can be initiated by many different stimuli including growth factor withdrawal, UV- or γ-irradiation, chemotherapeutic agents, heat shock, nutrient deprivation, or by a family of transmembrane proteins called death receptors (Figure 3). However, the apoptotic-inducing pathways in mammalian cells can be divided into the extrinsic and intrinsic pathways. The former is triggered by the external death signals, leading to activation of a caspase cascade (Figure 4) at intracellular receptor-signalling complexes, and is usually employed in the immune response.[7] The latter is triggered by

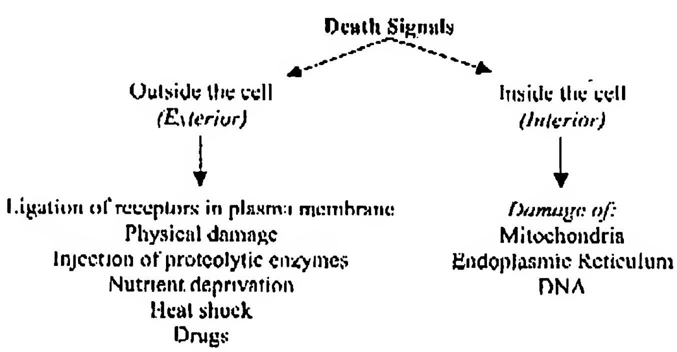

FIGURE 3 Caspases implicated in apoptosis and cancer in mammals.

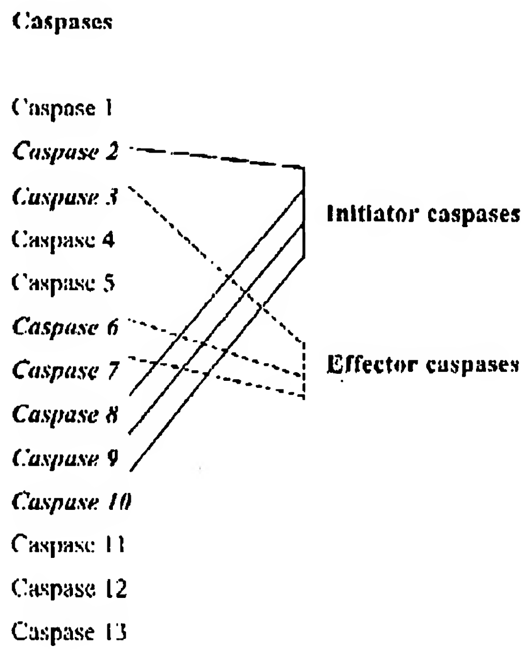

FIGURE 4 Death signals.

intracellular death signals and is activated when the cell is exposed to DNA-damaging agents. In this apoptotic pathway, the mitochondria play a central role, and the BCL2 family members are closely implicated in a regulatory role.

II. THE *BCL2* FAMILY OF PROTEINS

The members of the B-cell CLL/lymphoma 2 (*BCL2*) family are categorized into two main groups. The first group consists of the antiapoptotic members that share high structural and functional homology with *BCL2*, while the second includes proteins that share less homology to *BCL2* and display proapoptotic activity. The latter group is further divided into two subgroups, the BCL2-associated X protein (BAX)-like death factors and the BH3-only proteins.

The structural homology among the members of the family refers to from one-to-four regions that share high sequence homology to BCL2, designated as BH (BCL2-homology region) domains (BH1, BH2, BH3, BH4) and are common among the members of the family (Figure 5). These domains correspond to an α-helical configuration. Mutagenesis and deletion analysis studies have revealed that BH domains are important for function as well as for heterodimerization between the family members.[8–11] Indeed, the relevant interactions between the death-promoting and death-inhibiting proteins determine the susceptibility of the cell to the various apoptotic

BCL2 (BH1, BH2, BH3, BH4)
BCL2A1 (BH1, BH2, BH3, BH4)
BCL2L1 (BH1, BH2, BH3, BH4)
BCL2L2 (BH1, BH2, BH3, BH4)
BCL2L13 (BH1, BH2, BH3, BH4)
BCL2L10 (BH1, BH2, BH3, BH4)

BOO/DIVA (BH1, BH2, BH4)

MCL1 (BH1, BH2, BH3)
BAK1 (BH1, BH2, BH3)
BAX (BH1, BH2, BH3)
BOK (BH1, BH2, BH3)

BCL2L14 (BH2, BH3)
BFK (BH2, BH3)
BCL2L12 (BH2, BH3)

BAD (BH3)
BID (BH3)
HRK (BH3)
BCL2L11 (BH3)
BNIP (BH3)
BIK (BH3)
BLK (BH3)
PMAIP1 (BH3)
MAP-1 (BH3)
BMF (BH3)
BBC3 (BH3)

FIGURE 5 BH domains in BCL2 family members.

stimuli.[12] Furthermore, most of the members contain a hydrophobic carboxyterminal transmembrane (TM) domain that is probably responsible for their membrane localization.[13]

The antiapoptotic gene products are initially integral membrane proteins, localised mainly in the mitochondrial outer membrane. Furthermore, antiapoptotic molecules have been detected in the membranes of the endoplasmic reticulum (ER) and nucleus.[13–16] Their main role is to stabilise the mitochondrial membrane, preventing cytochrome c release and its subsequent binding to apoptosis activating factor-1 (Apaf-1).[17,18] By contrast, the proapoptotic BCL2 family members localise to cytosol or cytoskeleton, in a healthy cell. However, following a death signal, they usually interact with the antiapoptotic proteins, resulting in their inhibition and the initiation of the apoptotic machinery.

Since the antiapoptotic members are more conserved, they bear three-to-four BH regions, mediating their intracellular localization to the cytoplasmic side of internal membranes. The proapoptotic members of the family are less conserved, usually lacking the BH4 aminoterminal domain. However, the BH3 domain is highly conserved and is the most common feature of almost all family representatives. This amphipathic α helical domain must play a pivotal role in heterodimerization and death-promoting activity.[10,11,19] This concept is reinforced by the existence of a whole subcategory of proapoptotic genes called BH3-domain-only genes, which bear only the BH3 conserved domain. Deletion and/or site directed mutagenesis studies of some BH3-domain only proteins have revealed that absence of the BH3 region or modification of specific amino acids of this domain result in loss of their execution activity.

Studies suggest that the proapoptotic proteins must have their BH3 domain exposed constitutively or through post-translational modification in order to achieve killing function.[20,21] On the other hand, crystallography studies of BCL2 and BCL-X long form (BCL-X_L) demonstrate that BH1, BH2 and BH3 domains are found in close proximity, thus forming a hydrophobic cleft, which is stabilized by the N-terminal of the BH4 domain and interacts with the exposed BH3 α-helix region of a proapoptotic protein.[22] These results are consistent with their interaction properties. However, under physiological conditions, not all BH3-containing proteins can interact with the hydrophobic groove of the BCL2 survival factors. While pro-apoptotic BH3-only and BAX like proteins expose their BH3 domain after a post-translational modification and/or a conformational change, death-inhibiting BCL2 homologues maintain this domain as an integral part of their hydrophobic pocket. Their BH3 domains are not available for binding to other hydrophobic pockets of the BCL2 family members.

Overall, the relative ratio of pro-survival (BCL2-like) and proapoptotic (BAX-like and BH3-only) proteins seems to determine the cell sensitivity or resistance to the apoptotic stimuli.

A. Subgroup 1: The *BCL2*-Like Pro-Survival Factors

1. Antiapoptotic Members (Table 1)

a. BCL2 (Bcl 2). *BCL2* is a proto-oncogene that was identified at the chromosomal translocation breakpoint, between chromosomes 14 and 18, t(14;18), in non-Hodgkin's follicular B-cell lymphomas.[23–27] It promotes tumorigenesis by preventing cell death rather than by increasing the rate of cell division[28] and by arresting cells in the Go/G1 phase of the cell cycle. Its protein product is a 26 kDa protein consisting of 239 amino acids (human), with a single highly hydrophobic domain at its C-terminus,[29] which enables it to localise mainly in the mitochondrial outer membrane, and to a lesser extent in the nuclear envelope and the membrane of the endoplasmic reticulum.[16,30,31] The protein contains all four BH domains (BH1 to

TABLE 1 Antiapoptotic Members of the *BCL2* Family

Approved gene symbol	Approved gene name	Location	Aliases
BCL2	B-cell CLL/lymphoma 2	18q21.3	*Bcl 2*
BCL2L1 (transcript bcl-X_L)	BCL2-like 1	20q11.21	*BCLX, BCL2L, Bcl-X, bcl-xL, BCL-X_L, DKFZp781P2092*
MCL1 (transcript $MCL1_L$)	myeloid cell leukemia sequence 1 (BCL2-related)	1q21	*TM, EAT, MCL1L, MCC1839*
BCL2L2	BCL2-like 2	14q11-q12	*KIAA0271, BCL-W*
BCL2L10	BCL2 like 10 (apoptosis facilitator)	15q21	*BCL-B, Boo, Diva*
BCL2A1	BCL2-related protein A1	15q24.3	*GRS, BFL1, BCL2L5, HBPA1*

BH4).[32] BH1, BH2 and BH3 constitute the hydrophobic cleft through which the protein interacts and forms homo- and heterodimers with the proapoptotic members of the BCL2 family of proteins.

BCL2 is expressed in a wide variety of foetal tissues, whereas in adults it shows restricted expression in more rapidly proliferating and differentiating cells.[33] High levels of BCL2 have been detected in pro-B and mature B cells,[34,35] in most neurons of the developing mouse,[36] in thymus throughout the medulla but in fewer cells of the cortex,[37] in spleen[38] and in lymph nodes[38] as well as early in the embryonic kidney.[39] Decrease in its expression levels is observed in motor-neurons during the embryonic and postnatal periods[40] as well as in pre-B cells being prepared to differentiate.[41] It is characteristic that *BCL2* is expressed only in nonpregnant and early pregnancy mammary glands, with no expression in late pregnancy or involution.[42]

Expression of the protein inhibits apoptosis induced by various stimuli, such as chemotherapeutic drugs,[43] neuronal growth factor withdrawal in neurons,[44] or glucocorticoids.[45,46] *BCL2* gene can also be upregulated by interleukin-7 (IL-7)[47] and the tumour suppressor p53.[48] However, when the protein is regulated by phosphorylation at a Ser residue mediated by either the Ras/Raf/mitogen-activated protein (MAP) kinase[49] or the cysteine protease protein of molecular mass 32 kDa (CPP32) pathways,[50] it loses its antiapoptotic activity.[51] Consistent with the above, the phosphorylated form of BCL2 coimmunoprecipitates with the Ser/Thr specific kinase[52] as well as with p21Ras.

It has been reported that, in mammalian cells, BCL2 binds to Apaf1 (the human homologue of cell death protein-[CDP-4]),[53] sequestering caspase-9 and subsequently blocking the initiation of the proteolytic cascade.[54–56] However, the main role of BCL2 in the process of apoptosis is based on its ability to form ion conductive channels.[51–59] In most cases, BCL2 seems to act by preventing mitochondrial disruption and the release of cytochrome c, inhibiting the latter's association with Apaf1 and therefore the activation of caspase-9.[18] According to ongoing speculation, BCL2 may play a

role in the inactivation of initiator caspases, such as caspase-2, that act upstream or independently of cytochrome c release and mitochondrial involvement.[60]

b. BCL2L1 (BCLX, BCL2L, BCL-X). The BCL2-like 1 (*BCL2L1*) gene maps on chromosome 20q11.21 and consists of 3 exons and 2 introns, encoding a 233 amino acid protein, localised to the outer mitochondrial membrane. The first exon is untranslated while the first intron is facultative.[61] The gene undergoes alternative splicing, and, up to the present, three different splicing variants have been identified (*BCL-X$_L$*, *BCL-X$_S$*, *BCL-X$_γ$*).[62,63] BCL-X short form (BCL-X$_S$) derives from splicing of the second exon, which contains the BH1 and BH2 domains and has a length of 170 amino acids, while the large protein BCL-X$_L$ (233 amino acids) is encoded by exons 2 and 3.[61] These two proteins display striking functional and expressional differences. Thus, while the large transcript *BCL-X$_L$* is antiapoptotic and mainly expressed in long-lived cells, such as those of the nervous system, and localises in the perinuclear envelope and mitochondrial membranes,[64] the smaller protein BCL-X$_S$ is expressed in cells with a high turnover rate, such as those of the immune system, and acts as an apoptotic activator.[65,66] Another variant, designated as BCL-X gamma form (*BCL-Xγ*), has also been identified.[67] *BCL-X* is also widely expressed in the brain, kidney, thymus[66] and the physiological mammary gland, with its expression continuing during early and late pregnancy, but it is downregulated during lactation and upregulated again during the period of involution.[68] As far as the intracellular localization of BCL-X$_L$ is concerned, it is found both in cytosol and in intracellular membranes in healthy cells.[69]

BCL-X$_L$ protein is highly homologous to BCL2, containing the conserved domains BH1 to BH4 and a hydrophobic region at its C terminus. According to crystallography studies, the BH1, BH2 and BH3 motifs of BCL-X$_L$ are found in close proximity to one another, forming a hydrophobic pocket through which the protein interacts with the exposed BH3 region of proapoptotic molecules.[22] For example, the presence of an antiapoptotic molecule, such as BCL2 or BCL-X$_L$, can inhibit the activation of the death promoter BAX.[70] Thus, BCL-X$_L$, like all the antiapoptotic members of the BCL2 family, must regulate apoptosis via interaction with and blockage of the proapoptotic proteins.

BCL-X$_L$ presents a three-dimensional structure in which the two central hydrophobic cores are surrounded by four amphipathic helices. This structure is similar to the pore-forming regions of bacterial toxins that form ion channels.[21,71] This is consistent with the ability of BCL-X$_L$ to form ion conductive channels *in vitro*.[22,57,58,72] All the above data suggest that BCL-X$_L$ may regulate survival by regulating the permeability of the intracellular membranes,[72] preventing the removal of the cytochrome c in cytosol, and preserving the membrane integrity. On the other hand, it has been reported that BCL-X$_L$ binds to Apaf-1, thus forming a complex that prevents

the activation of caspase-9. This complex can also be disrupted by some death-inducing molecules.[54,73,74]

The smaller counterpart, BCL-X_S, is a BH3-domain-only protein lacking the hydrophobic α helices 5 and 6, which are essential for membrane insertion. Therefore, it provokes apoptosis through binding to the hydrophobic pocket of BCL2 or BCL-X_L to inhibit their antiapoptotic activity.

The *BCL-X* gene is transcriptionally responsive. Its expression is up-regulated by glucocorticoids and irradiation,[75] by IL-3 and insulin-like growth factor-1 (IGF-1), through the phosphatidylinositol 3-kinase (PI3K) pathway,[76] by nerve growth factor (NGF) treatment,[77] through the cluster of differentiation (CD40)/CD40 ligand (CD40L) pathway,[78] and by IL-2 through the C28 receptor pathway.[79]

c. MCL1 (TM, EAT, MGC1839). *MCL1* (myeloid cell leukaemia 1) is an antiapoptotic member of the *BCL2* family. Its cDNA has a length of about 3.8 kb and encodes for a protein with a molecular mass of 37.3 kDa consisting of 350 amino acid residues.[80] The protein is highly homologous to BCL2, containing BH1, BH2 and BH3 domains as well as a hydrophobic transmembrane region at its C-terminus.[81] Furthermore, the N-terminus of the protein contains two regions rich in the glycine/alanine motif, two sequences enriched in proline/glutamic acid/serine and threonine, also known as PESTs,[82] and repeated pairs of arginines. The last two features are characteristic of a variety of other proteins implicated in tumorigenesis. The gene consists of 3 exons, 2 introns and an untranslated region of 370 bp.[81,83]

MCL1 expression is tightly regulated at transcriptional, post-transcriptional and post-translational levels. As far as the transcriptional level is concerned, exposure of hemopoietic cell lines to phorbol-12-myristate-13-acetate (TPA) leads to the activation of the extracellular signal-regulated kinase (ERK)-mediated pathway, which results in the binding of a transcription factor complex (serum factor [SRF]; Elk-1) to *MCL1* promoter.[84,85] This ultimately increases *MCL1* transcriptional levels. Other pathways that also increase *MCL1* transcriptional levels are the MAP kinase-mediated pathway (MAPK/ERK or p38),[86–88] PI3K/Akt[89] and Janus kinase (JAK) signal transducers and activators of transcription (STAT3) transactivation pathways.[90–93]

MCL1 may also be regulated post-transcriptionally by alternative splicing, resulting in the production of $MCL1_S$ (MCL1 short form). The splice variant, which has been found in human placenta,[81] derives from the association of the first and third exons and the elimination of the second one. $MCL1_S$ lacks regions BH1, BH2 and the hydrophobic C-terminal region, while its BH3 domain remains constant in comparison to the full-length molecule $MCL1_L$.[81] Thus, $MCL1_S$ displays features similar to the proapoptotic BH3-domain-only proteins, with its overexpression provoking cell death in Chinese hamster ovarian cells[81,94] and its possessing antagonistic activity to $MCL1_L$, yet, being able to dimerize with it.[81] Therefore, while $MCL1_S$ possesses proapoptotic activity, $MCL1_L$ possesses antiapoptotic activity. MCL1

post-translational modifications involve MCL1 protein phosphorylation, occurring through two distinct pathways, one TPA-induced ERK-dependent and the other taxol-induced or okadaic acid induced, with only the latter phosphorylation pathway ultimately resulting in the electrophoretic mobility shift of the MCL1 protein.[51,95–101]

MCL1 initially had been isolated from a myeloid leukemia cell line (ML-1), although it has been found to be widely expressed *in vivo*.[102] It is normally expressed in a cell type-specific manner in response to signals that affect cell growth, differentiation and cell viability.[103] Its antiapoptotic function and short viability-promoting activity was evident after transfection of murine myeloid progenitor cells (FDC-P1), which resulted in enhanced survival under apoptotic-inducing conditions, such as UV-irradiation, withdrawal of required growth factors and exposure to a variety of chemotherapeutic agents.[104,105] Consequently, decreased expression of *MCL1* is associated with cell death induced by agents such as sodium salicylate and etoposide.[106] Exposure of human B cells to the cytokine IL-13 results in MCL1 stimulation, which inhibits apoptosis in human B cells, leading to their proliferation. This inhibition is reinforced through the interaction of the latter with the survival factor CD40L, inducing the expression of *MCL1* via the CD40L/CD40 pathway.[107,108] Other survival factors, namely IL-6 and interferon-alpha (IFN-α), rapidly up-regulate MCL1.[109,110] Moreover, functional analysis studies of the *MCL1* gene revealed the presence of regulatory sequences that show homology to phorbol ester (PMA) and granulocyte macrophage colony-stimulating factor (GM-CSF).[83] Indeed, MCL1 is regulated via the GM CSF signalling pathway. Using the TF-1 myeloid progenitor cell line, it was shown that MCL1 levels were reduced immediately after the withdrawal of this survival factor (*i.e.*, factors whose overproduction can delay apoptosis induced by various agents), an absolutely reversible phenomenon.[111] According to another study, human neutrophils that are highly susceptible to apoptosis survive after treatment with cytokines such as GM-CSF or IL-3β. Thus, cellular levels of MCL1 in neutrophils are enhanced after cytokine treatment and declined following apoptosis induction.[112]

Increased *MCL1* expression also serves to provide additional viability in a broad range of cell types, including hematopoietic cells of various lineages. Several studies showed that, although *MCL1* is not expressed in mature cells, it is expressed in immature cells of myeloid and erythroid lineages as well as in differentiating ML-1 cells *in vitro*.[103] *MCL1* is also expressed at specific stages in lymphoid cell differentiation, with its expression being low in memory B cells, which can live for an extended period of time, but prominent in germinal center B cells, which undergo affinity maturation and die through apoptosis.[103] Therefore, the expression of *MCL1* in germinal center B cells may serve to promote viability for a short period of time to allow the selection of clones exhibiting the appropriate antibody affinity and ensuring the rapid elimination of the remaining cells.[103] *MCL1* is also expressed in plasma cells,

large activated lymphocytes, and epithelial tissues. *MCL1* expression is low in less differentiated cells (basal cells) of epithelial tissues, whereas it is highly expressed in more differentiated ones (upper layers).[103]

d. BCL2L2 (BCLW, BCL-W, KIAA0271. The BCL2-like 2 (*BCL2L2*) gene maps on human chromosome 14q11.[113] Its encoding protein product is an antiapoptotic, highly conserved BCL2 family protein. It consists of 193 amino acids and bears all the conserved BH domains (BH1 to BH4) and a hydrophobic C-terminal transmembrane region (TM).[113] Since BCL-W contains the conserved domains BH1 to BH4, it can interact with other proteins of the family to modulate apoptosis and has been found to co-immunoprecipitate with proteins such as BAX, BCL2-antagonist of cell death (BAD), BCL2-antagonist/killer (BAK) and BCL2-interacting killer (BIK).[114]

The protein plays a pivotal role in the mature nervous system[115] as well as in spermatogenesis, since *BCL-W -/-* adult male mice are unable to produce mature sperm.[116,117] It also aids the survival of epithelial cells in the gut. In addition, it is expressed mainly in cells of myeloid, lymphoid and epithelial origin,[118] including those of the mammary gland, and is up-regulated at the beginning of involution. At the intracellular level, it localises at mitochondrial membranes, where it is associated loosely in healthy cells and tightly in apoptotic cells, losing its antiapoptotic activity.[119–121]

The protein is regulated at the transcriptional level via the extracellular death signal mechanism of apoptosis. Some stimuli that upregulate the gene's transcription are follicli-stimulating hormone (FSH), testosterone withdrawal,[122] and the protooncogene met.[123] The two latter stimuli provoke the elevation of BAX/BCL-W and BAK/BCL-W ratios in cells involved in spermatogenesis, contributing to their survival by blocking the apoptotic effect of BAX and BAK.[122] Met causes BCL-W overexpression, leading to colorectal tumour development.[123] L-Ser and Gly can also contribute to neuronal survival by the up regulation of the BCL-W gene.[124] BCL-W promotes cell survival by preventing the release of cytochrome c.[125]

e. BOO/DIVA. The human BCL2 homologue of the ovary *(BOO)*/death inducer binding to vBCL2 and apoptosis-activating factor, APAF-1 (DIVA) gene has been mapped on human chromosome 15q21[126] and mouse chromosome 9d9[73] and encodes for a protein that consists of 191 amino acid residues, with a molecular mass of 22.3 kDa. BOO/DIVA is a BCL2 family member that was initially isolated because of its high homology to the chicken antiapoptotic protein NR13. It displays a very restricted tissue distribution since it is detected only in the ovary, testis and the epidydimis of adult mice,[73,74] showing a wide distribution in mouse embryo,[126] whereas in humans it is expressed in kidney, liver and ovary.[126] It contains the BH1, BH2 and BH4 conserved domains and a hydrophobic C terminal region, suggesting that it is an integral membrane protein, whereas lacking the BH3

motif makes it unable to heterodimerize with the other members of the family. BOO/DIVA binds to Apaf-1, and this is the mechanism by which it mediates apoptosis. It has been reported that Apaf-1, the mammalian homologue of CED 4 from *C. elegans*, interacts with BCL-X_L and caspase 9 to form a ternary complex. This complex blocks the caspase activity, thus contributing to cell survival. BOO/DIVA also interacts with Apaf-1, contributing to the regulation of apoptosis in the same way.[73,74] Proapoptotic members such as BAK, BAX and BIK provoke apoptosis with the dissociation of the complex and the release of caspase 9. In addition, BOO/DIVA has been reported to acquire proapoptotic or antiapoptotic activity, according to the cell type.[73,74,126]

f. BCL2A1 (BFL1, BCL2L5, GRS, HBPA1, BCL2A1). BFL1 is a human gene, cloned from fetal liver and endothelial cells cDNA libraries, as a tumour necrosis factor (TNF)-inducible transcript, using degenerate primers complementary to two conserved regions of the BCL2 family.[127] The human gene maps on chromosome 15q24-25[128] and codes for a 175 amino acid protein. The BFL1 protein protects endothelial cells against TNF-induced apoptosis.[129] Its mouse counterpart, BCL2A1, was first identified from GM-CSF- induced mouse bone marrow as a novel hemopoietic-specific gene, with sequence similarity to BCL2 and a 72% amino acid identity to the human BFL1. BFL1/BCL2A1 contains all four of the conserved domains BH1 to BH4, missing, however, a well defined C-terminal TM domain, which is characteristic of most BCL2 family proteins.[130]

BFL1 mRNA has a short half-life and is expressed abundantly in bone marrow, spleen, lung and at low levels in various cancer cell lines, thymus, testis and small intestine.[127,131] It has recently been demonstrated that mice have three functional isoforms of BCL2A1 (a, b and c) that are probably derived from gene duplication,[132] whereas in humans at least two isoforms of BFL1 were found, one resulting from the exclusion or inclusion of a 56-base pair exon (alternative splicing) of the *BFL1* gene, designated as BFL short form ($BFL1_S$).[133] $BFL1_S$ is a novel human BCL2 family member that targets the nucleus with its C-terminal nuclear localization signal (NLS).[133]

Expression of *BFL1/BCL2A1* appears to be induced by inflammatory cytokines, TNF and IL-1β,[127,134] PMA,[127] lipopolysaccharide (LPS),[135] pathogens,[136–138] vascular endothelial growth factor (VEGF),[139] GM-CSF,[140] granulocyte colony-stimulating factor (G-CSF),[139,140] CD40L[141] and the anticancer drug etoposide,[142] which share the capacity to activate the nuclear factor kappa B (NF-κB) which regulates BFL1/BCL2A1 expression.[143,144] BFL1/BCL2A1 can strongly dimerize with BAX, thus inhibiting the release of cytochrome-c and caspase 3 activation and suppressing apoptosis induction.[145–147] Therefore, this probably involves binding and inactivation of pro-apoptotic proteins, such as the BH3 domain-only protein BID and the multidomain protein BCL2-related ovarian killer (BOK).[148]

TABLE 2 Proapoptotic Members of the *BCL2* Family

Approved gene symbol	Approved gene name	Location	Aliases
BAX	BCL2-associated X protein	19q13.3–q13.4	*Bax zeta*
BAK1	BCL2-antagonist/killer 1	6p21.3	*BCL2L7, BAK, CDN1*
BAD	BCL2-antagonist of cell death	11q13.1	*BCL2L8, BBC2*
BOK	BCL2-related ovarian killer	2q37.3	*BCL2L9, BOKL, MGC4671*
BID	BH3-interacting domain death agonist	22q11.1	*MGC15319, MGC42355*
HRK	harakiri, BCL2 interacting protein (contains only BH3 domain)	12q24.2	*DP5*
BCL2L11	BCL2 like 11 (apoptosis facilitator)	2q11.2–2q14.3	*BOD, BimL, BimEL*
BNIP1	BCL2/adenovirus E1B 19 kDa interacting protein 1	5q33–q34	*Nip1*
BNIP2	BCL2/adenovirus E1B 19 kDa interacting protein 2	15q21.3	*Nip2, BNIP-2*
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	10q26.3	*Nip3*
BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	8p21	*Nix, BNIP3a*
BNIP3P	BCL2/adenovirus E1B 19 kDa interacting protein 3 pseudogene	14q12	
BNIPL	BCL2/adenovirus E1B 19 kD interacting protein like	1q21.2	*BNIPL1, BNIPL2, PP753*
BIK	BCL2-interacting killer (apoptosis-inducing)	22q13.31	*NBK, BBC1*
BLK	B lymphoid tyrosine kinase	8p23–p22	*MGC10442*
PMAIP1	Phorbol 12 myristate 13 acetate-induced protein 1	18q21.32	*NOXA, APR*
BMF	Bcl2 modifying factor	15q14	
BCL2L1 (transcript bcl-Xs)	BCL2-like protein 1	20q11.21	*BCLX, BCL2L, Bcl-X, bcl-Xs, BCL-Xs, DKFZp781P2092*
MCL1 (transcript MCL1S)	myeloid cell leukemia sequence 1 (BCL2-related)	1q21	*TM, EAT, MCL1S, MGC1839*
BBC3	BCL2 binding component 3	19q13.3–q13.4	*JFY1, PUMA*

B. Subgroup 2: BAX-Like Death Factors

1. Highly Conserved Proapoptotic Genes (Table 2)

a. BAX (Bax zeta). Somatic cell hybrid panels and *in situ* hybridization[149] indicated that the human *BAX* gene maps on chromosome 19q13.3–q13.4. The *BAX* gene consists of 6 exons and 5 intervening introns encoding for a 21 kDa protein, named BAXα (alpha). The latter, usually called BAX, presents high homology to the BCL2 protein, containing the conserved regions BH1, BH2 and BH3, enabling it to heterodimerize with BCL2 and to display its proapoptotic function.[8] The formation of heterodimers between BAX and the other members of the BCL2 family is implicated in

the regulation of the apoptotic mechanism.[12] However, a recently identified cytoplasmic BAX-binding protein, BIF-1, lacks any of the already known features of the BCL2 family members, and instead contains an Src homology 3 (SH3) domain near its C-terminus.[150] Its BAX-binding activity implies that BAX can mediate apoptosis via indirect interactions with tyrosine kinases.

The BH3 domain of BAX is essential for its homodimerization and its heterodimerization with BCL2 and BCL-X_L.[151] Furthermore, the protein contains a hydrophobic C-terminal region essential for membrane targeting, while BH1 and BH2 domains show homology to pore-forming proteins that contribute to apoptosis.

In addition to producing the major BAXα protein, which is the translational product of the whole gene, the latter undergoes alternative splicing, resulting in the production of the proteins BAX-β, BAX-γ and BAX-δ. Unlike BAX-β and BAX-δ, which are cytosolic proteins, BAX-δ lacks the peptide region encoded by exon 3, whereas it retains the BH1 and BH2 domains as well as the hydrophobic C-terminal region. Another BAX isoform, BAX-σ, has been recently identified. BAX-σ contains BH1, BH2 and BH3 domains, the α-5 and α-6 helices, and also the C-terminal hydrophobic transmembrane domain, but lacks the amino acid residues 159 to 171.[152]

The tumour suppressor p53 seems to be a regulator of BAX at the transcriptional level. Indeed, it has been proved that the promoter of the BAX gene bears four regions with high homology to the consensus p53 binding sites.[153] On the other hand, *in vitro* experiments have shown that p53 up-regulates the BAX gene, while *in vivo* tissues of p53 -/- mice showed decreased levels of the protein.[48]

BAX is the first death-promoting member of the BCL2 family to be identified, and it was detected as a protein co-purified with BCL2 in immunoprecipitation studies.[154] Its proapoptotic function was proved when overexpression of BAX in an IL-3 dependent cell line accelerated apoptosis in the absence of IL-3. Intracellular localization studies demonstrated that BAX is initially a cytosolic monomeric protein. Following exposure to a death stimulus, however, it translocates to mitochondria and becomes an integral membrane protein that can be cross-linkable either as a homodimer or a homomultimer.[70,155] This occurs because BAX is predominantly cytosolic in a conformation in which its N-terminal helix-1 is hidden. Upon proapoptotic signalling, BAX undergoes a conformational change that exposes its N terminus and, possibly, its BH3 domain.[156,157] This allows its translocation and its tight association with the mitochondria, through integration into the outer membrane and formation of homo-oligomers and hetero-oligomers with BAK.[158–160] Some controversial data suggest that BAX interaction with cardiolipin (a mitochondria-specific lipid) is sufficient to trigger mitochondrial membrane potential (MMP),[161] or that it interacts (directly or indirectly) with mito chondrial proteins, voltage-dependent anion channel (VDAC),[162] and the

adenine nucleotide translocase.[161,163–166] In any case, it is likely that BAX oligomerization and tight association with the outer mitochondrial membrane lead to MMP,[161,164–167] inducing homodimerisation of BAX protein.[168]

The presence of antiapoptotic molecules such as BCL2 and BCL-X_L, can inhibit the activation of BAX following a death signal.[70] However, the killing function of BAX is not associated with its ability to interact with these molecules. BAX mediates apoptosis through a mitochondrial mediated pathway that can be either caspase-associated or not, with the caspase-dependent mitochondrial pathway being based on the release of cytochrome c from the mitochondrial membranes. After a variety of death signals, proapoptotic molecules translocate to membranes where the antiapoptotic proteins already reside. Thus, the dimerization of BAX provokes changes in the mitochondrial membrane, such as an alteration at the membrane potential $\Delta\Psi_m$ and release of cytochrome c. The latter, after exposure to cytosol, forms a complex with Apaf-1, which, in turn, recruits the initiator caspase-9. The activation of the latter leads to a proteolytic cascade and to cell disruption.[58,70,169–172] The involvement of BAX in the release of cytochrome c from the mitochondrial membrane is consistent with its ability to form ion-conductive pores in artificial lipid bilayers *in vitro*, and this leads to the release of synthetic substrates.[59]

BAX has been reported to interact with porin and to form large pores (VDAC).[62] Outer membrane proteins such as porin, in association with inner membrane proteins such as adenine nucleotide transporter (ANT) and other proteins such as adenylate kinase and hexokinase, compose a multimeric complex that forms a large pore, called permeability transition pore (PTP). BAX co-immunoprecipitates with PTP,[173] implying that this possible interaction increases mitochondrial permeability. However, this suggestion is controversial because it has been found that cytochrome c release occurs even in the absence of PTP.[174] When the dimer enters the membrane, it undergoes a conformational change by protruding its N-terminal region into the cytosol.[175] The physiological purpose of this is to keep the molecule in a close conformation until an apoptotic stimulus appears.

On the other hand, BAX can mediate apoptosis through a caspase-independent pathway. Indeed, Xiang *et al.*[169] and McCarthy *et al.*[176] demonstrated that BAX could induce apoptosis in conditions that cause broad caspase inhibition.

Important though it may be for apoptosis, the mechanism of BAX regulation is poorly understood. Regulation of BCL2 family members can occur by a number of mechanisms. Lewis *et al.*[177] have demonstrated that recombinant BAX lacking the C-terminal tail is phosphorylated *in vitro* both by MAP kinase 1 (MAPK1) and protein kinase A (PKA), and that Ser184 is important for the regulation of BAX activity. Its phosphorylation requires PI3K protein kinase B (Akt) activation and appears to be mediated by Akt itself. In its phosphorylated form, BAX was detected in the cytoplasm and

promoted heterodimerization with MCL1, BCL-X_L and BCL2A1. Apoptotic neutrophils possessed reduced levels of phosphorylated BAX, correlating with an increase in activated BAX as well as an increase in the amount of BAX found to have been translocated to the mitochondria. It has also been suggested that such a phosphorylation inhibits the effects of BAX on the mitochondria by maintaining the protein in the cytoplasm, heterodimerized with antiapoptotic BCL2 family members. There are also reports indicating that BAX function is altered in chronic inflammatory diseases and that, under these conditions, neutrophil apoptosis is significantly attenuated.[178]

BAX is a protein important for the control of cell death. Cells that overexpress BAX show enhanced apoptosis,[179] whereas *BAX*-null cells show resistance to apoptosis. BAX expression has also been associated with tumour development and hematopoietic malignancies.[15,180]

b. BAK1 (BCL2L7, BAK, CDN1). BCL2 homologous antagonist/killer 1 (*BAK1*) has been cloned as a *BCL2*-related gene, which consists of 6 exons and maps to chromosome 6p21.3 on the human genome,[181,182] encoding a 211-amino acid protein with a relative molecular weight (*Mr*) of 23,400. It is a death promoting member of the BCL2 family, structurally and functionally very similar to BAX, and contains the conserved domains BH1 to BH3 and a hydrophobic core in its C-terminus. BAK presents a widespread tissue distribution,[183] and its regulation takes place at the transcriptional level, with overexpression of p53,[184,185] or *in vitro* TNF-γ treatment,[172,186] up regulating the expression of the gene.

The antiapoptotic member BCL-X_L interacts with BAK,[187] inhibiting its activity.[172] However, since BAK has a similar structure to BCL-X_L, with the BH1, BH2 and BH3 domains forming a hydrophobic pocket that prevents the BH3 domain from gaining access to the hydrophobic pocket of BCL-X_L,[187] a conformational alteration must take place in order for BAK to proceed to heterodimerization.[11]

The function of BAK has been correlated with the proapoptotic truncated BH3 interacting domain death agonist protein (tBID). Indeed, *BAK*-deficient mitochondria are unable to release cytochrome c when they are exposed to tBID.[188] On the other hand, BAK, in coordination with tBID, a death ligand that binds to BAK, can trigger cytochrome c release through mitochondrial pore formation. Its Fas/FasL-mediated activation leads to translocation into the mitochondrial membrane. There BAK, which is allosterically responsive to tBID, produces homodimers or homo-oligomers, causing essential changes in mitochondrial membrane potential and inducing the release of cytochrome c, which subsequently initiates the Apaf-1 proteolytic cascade.[172,176] It is important that BAK does not enhance mitochondrial pore permeability transition in this mechanism.[188,189]

BAK can mediate apoptosis in an additional caspase-independent pathway.[176] In agreement with this, cells lacking BAX and BAK did not respond to apoptotic stimuli that act via mitochondrial membrane disruption,

such as the chemotherapeutic drug etoposide, growth factor withdrawal, and exposure to UV.[190]

Sun *et al.*[191] identified and characterized N-BAK, a neuron-specific isoform of BAK. N-BAK is generated by neuron-specific splicing of a novel 20-base pair exon, which changes the previously described BAK, containing BCL2 homology domains BH1, BH2 and BH3, to a shorter BH3-domain-only protein. N-BAK transcripts are expressed only in central and peripheral neurons, but not in any other cells, whereas BAK itself is expressed ubiquitously, except in the neurons. Neonatal sympathetic neurons microinjected with N-BAK have been shown to resist apoptotic death caused by NGF removal, whereas microinjected BAK accelerated NGF deprivation-induced death. Overexpressed BAK killed sympathetic neurons in the presence of NGF, whereas N-BAK did not. N-BAK was, however, still death promoting when overexpressed in non-neuronal cells. Thus, N-BAK is an antiapoptotic BH3 domain-only protein but only in the appropriate cellular environment.

c. BOK (BCL2L4, BOKL, MGC4631)/MTDE (MTD, MT1). *BOK* was first identified from a rat fusion ovarian cDNA library due to its heterodimerization with the antiapoptotic *MCL1*,[192] whereas its mouse homologue (*MTD*) was identified from a mouse gene database.[192] BOK has been predominantly detected in all reproductive tissues in rats, such as ovary, testis and uterus,[192] suggesting an important role in the reproductive system, whereas MTD is mostly expressed in lymphoid tissues.[192,193] It consists of 213 amino acids and possesses a predictable molecular mass of about 23.5 kDa.[192,193] BOK/MTD shows high homology to BCL2 since it bears the BH1, BH2 and BH3 conserved motifs and a hydrophobic C-terminal TM domain, and lacks the BH4 domain, like the other proapoptotic members of the family. Furthermore, BOK contains two potential phosphorylation sites at its N-terminus that could play a regulatory role.

BOK/MTD possesses the unique ability to interact selectively with some of the antiapoptotic members of the family. Thus, unlike BAX and BAK, it does not interact with BCL2 or BCL-X_L[192,193] but only with MCL1, BFL1 and the Epstein-Barr-virus-derived BHRF-1.[192] These results suggest that BOK interacts with MCL1 in reproductive cells to regulate apoptosis. Indeed, MCL1 is highly expressed in the ovary, whereas BCL2 is not.[192] The common localization of BOK, NOXA and MCL1 in mitochondria further supports the interaction of BOK with MCL1 as well as with NOXA.[194–196]

The presence of caspase inhibitors such as crmA did not suppress the BOK/MTD killing activity. On the other hand, an *MTD* mutant, with substitutions of highly conserved amino acid residues in the BH3 domain, retained its killing activity, while another *MTD* mutant, lacking BH1, BH2 and COOH domains, was unable to provoke apoptosis.[193] All the above data suggest that BOK/MTD mediates apoptosis via heterodimerization with antiapoptotic proteins[197] or by disruption of the mitochondrial membrane,[57] as is the case with BAX and BAK.

mRNA and protein expression analysis demonstrated that treatment of SH-SY5Y cells with etoposide resulted in p53-dependent induction of the *BOK* gene, which preceded or coincided with cytochrome-c release.[198] No induction of BOK or cytochrome-c release was detected in etoposide-treated MCF-7 cells,[198] but intrinsic DNA damage causes expression of BOK in MCF-7 cells, resulting in the release of cytochrome c to the cytosol without external treatment.[199] Inhibition of *BOK* gene expression protected germ cells from apoptosis. During testicular development, the highest expression of *BOK* mRNA occurs at the time of apoptosis. According to recent data, *BOK* expression increases with development as apoptosis increases over time in cell aging.

Two isoforms of *BOK* gene have been identified as a result of alternative splicing, namely the wild-type (WT) isoform BOK, which is found mainly in the mitochondria, and the smaller splice variant BOK-S, predominately found in the cytosol.[197] This splice variant derives from the splicing out of exon 3, retaining the BH2 and hydrophobic TM domains, but BH1 and BH3 domains are not affected by the process of splicing. BOK-S is unable to dimerize, although it can still promote death, implying that it stimulates apoptosis by damaging the intracellular membranes.[197] Overexpression of cytosolic BOK in a bursal lymphoma-derived cell line, DT40, induced apoptosis through a mitochondrial-dependent pathway, whereas the wild type isoform of BOK had a reduced phenotype in these cells.[200] In contrast, overexpression of BOK resulted in extensive apoptosis in myc-induced pre-neoplastic bursal cell populations but not in tumour cells.

C. Subgroup 3: The Pro Apoptotic BH3-Only Members of the BCL2 Family

1. Proapoptotic BH3-Domain-Only Members (Table 2)

a. BAD (BCL2L8/BBC2). The BCL2-antagonist of cell death *BAD* gene maps to chromosome 11q13.1 and encodes for a protein product of 168 amino acids. BAD (BCL-X_L/BCL2-associated death promoter) is a proapoptotic member of the BCL2 family of proteins that was initially detected because of its ability to interact with the anti-apoptotic proteins BCL-X_L and BCL2. On the other hand, it neither heterodimerizes with other members of the family, such as proapoptotic BAX, BCL-X_S, antiapoptotic MCL1 and BFL1/BCL2A1, nor homodimerizes.[201] BAD dimerization with BCL-X_L and BCL2 resulted in inactivation of BCL-X_L but not in that of BCL2.[201] The BAD protein presents minor homology with the BCL2 protein since it contains only the BH3 domain. Similar to the other members of the family, the BH3 domain is essential for its heterodimerization with the other family members.[19] Northern blot analysis demonstrated that the BAD protein presents a wide tissue distribution but is expressed at higher levels in lung, ovary, uterus and brain.[202]

BAD was the first BH3 domain only molecule to be associated with signal transduction through its differential phosphorylation in response to extracellular survival factors.[11] Dephosphorylated BAD appears to be active and bound to BCL2 and BCL-X_L in the mitochondria, whereas, when phosphorylated on serine residues (Ser-75, -112, -136, -155 and -170), it is inactive,[203–209] resides in the cytosol, and can be bound to 14-3-3 protein.[210] The cleavage of 14-3-3 protein during apoptosis promotes cell death by releasing the associated BAD and facilitating BAD translocation to the mitochondria and its interaction with BCL-X_L.[210] Factors including IL 3, IGF-1, platelet-derived growth factor (PDGF), and NGF transduce intracellular survival signalling mediated by activation of kinase cascades, resulting in the phosphorylation of death substrates, including BAD.[211–213] Accordingly, transgenic mice carrying BAD mutants lacking phosphorylation sites exhibit defects in growth factor-dependent survival.[213] Thus, the reversible phosphorylation of BAD represents a critical sensor for survival signalling and a determinant for the outcome of apoptotic stimuli.

A number of different Ser/Thr protein kinases are known to phosphorylate BAD at different Ser residues in the protein. The most-studied phosphorylation sites are found at serine residues 112, 136 and 155 in the mouse protein[211,213–215] and Ser-75, -99 and -118 in the corresponding human protein.[214] Recently, one more site, Ser-170, has also been shown to be phosphorylated in response to growth factors, preventing cytotoxic effects of BAD.[203–208] BAD can be phosphorylated by different kinases, in particular, Akt/PKB at Ser-136.[215,216] BAD can be phosphorylated at Ser 112 by ribosomal S6 kinase 1/2 (RSK 1/2), PKA and p-21-activated kinase (PAK).[217–219] PKA and RSK1 can phosphorylate BAD at Ser-155.[203–207] Cyclic AMP (cAMP)-dependent PKA phosphorylates BAD at murine Ser-112 and 155, while PI3 k/Akt (PKB) phosphorylates murine BAD at Ser-136.[203,220] In addition, human BAD can be phosphorylated at Ser-75 by Rac1.[209] The most recently identified BAD kinases are PAKs. While PAK1 and PAK2 were shown to phosphorylate murine BAD at both serines 112 and 136,[221,222] PAK4 and PAK5 only phosphorylate murine BAD at Ser-112.[223,224] BAD is also phosphorylated at Ser136 by Akt kinase, both *in vivo* and *in vitro,* blocking apoptosis induction.[225,226] PKA resides in the mitochondrial membrane in the presence of survival factor IL-3. After deprivation of IL-3, the enzyme is activated and phosphorylates BAD specifically at Ser112.[227] PIM-2 (34 kDa) kinase does not appear to regulate expression of BCL2, BCL-X_L, BCL2 interacting mediator (BIM), or BAX proteins. However, this kinase can phosphorylate BAD on Ser 112, which accounts in part for its ability to reverse BAD-induced cell death.[228]

Overexpression of *BAD* mRNA in granulosa cells induced apoptosis, which was reversed after treatment of the cells with a caspase inhibitor.[202] Thus, we can say that BAD mediates apoptosis via a caspase-dependent pathway. Evidently, the association of BAD/BCL2/BCL-X_L results in the release

of cytochrome c, which, in turn, activates the proteolytic caspase-mediated cascade.[202]

It has also been shown that overexpression of full-length, wild-type BAD (wt BAD) sensitizes LNCaP cells (a prostatic carcinoma cell line, resistant to TNF-related apoptosis-inducing ligand [TRAIL]-induced apoptosis) to TRAIL-induced apoptosis, with subsequent release of cytochrome c and Smac/DIABLO from mitochondria.[229] In this apoptotic pathway, wt BAD is cleaved in a caspase-dependent manner that results in generation of a protein with Mr of 15,000. However, LNCaP cells, expressing truncated BAD (tBAD) or BAD mutated at the caspase cleavage site, were less sensitive to TRAIL treatment when compared to LNCaP expressing wt BAD. Furthermore, differences in phosphorylation of serine residues for wt BAD and tBAD were identified, revealing that BAD-mediated sensitivity of LNCaP cells to TRAIL depends on the phosphorylation status of both wt BAD and tBAD.[229]

In contrast to its known proapoptotic function, it has also been reported that endogenously overexpressed BAD can inhibit cell death in neurons and other cell types.[230] The mechanism responsible for the functional conversion of BAD from an antiapoptotic to a proapoptotic factor involves alternative splicing that produces the N terminally truncated BAD_S (short form).[230]

b. BID(MGC15319, MGC42355). The *BID* gene is localised on chromosome 22q11.1, encoding a protein of 195 amino acids with a predicted molecular mass of 21,95 kDa in humans.[231] It belongs to the BH3-only domain proteins, and it was first cloned from a cDNA library via its interaction with BCL2 and BAX.[231] It does not contain a C-terminal hydrophobic region, thus it is considered a cytosolic protein. Three-dimensional structure studies revealed that BID displays a high similarity to $BCL\text{-}X_L$ protein in terms of its tertiary structure.[21,22,71]

BID has a unique role in apoptotic signalling because it links the death receptor signalling pathway to the mitochondrial signalling pathway, mediated by the BCL2 proteins. Death receptor binding activates caspase-8, which cleaves BID at Asp59 in its N-terminal region. The remaining free C-terminal fragment of BID, also known as p15 or tBID, translocates to the outer mitochondrial membrane, being implicated in the release of cytochrome c and the induction of apoptosis.[20] tBID is more potent in inducing cell death or interacting with BCL2 than the predominant protein.[232] BID and BAX cooperate to provoke mitochondrial membrane dysfunction.[233] One proposed model is that BID reaches the mitochondrial membrane to inactivate BCL2 or activate BAX.[231]

BID p15, similarly to BAX, has been shown to form ion conductive pores *in vitro*.[234] BID, after translocation to the mitochondrial membrane, provokes the release of cytochrome c.[20,170,235] The release of cytochrome c has been shown to promote the formation of a cytochrome c/Apaf-1/caspase-9 complex that activates the latter and results in the cleavage of the effector caspases 3 and 7.[53,170,236] Two different theories have been developed

for the precise mechanism by which cytochrome c exits the mitochondrial membrane. tBID could release cytochrome c indirectly by activating other membrane proteins. On the other hand, tBID as well as BAX may cause a number of other mitochondrial events leading to the disruption of the membrane and the subsequent execution of the cell, including decrease in the membrane potential $\Delta\psi_m$.[237]

Three novel isoforms of BID have been identified, BID_S, BID_{EL} and BID_{ES}. BID_S contains the N-terminal regulatory domain of BID, missing the BH3 domain; BID_{EL} corresponds to full-length BID; and BID_{ES} contains the BID sequence downstream of the BH3 domain.[238] Expression of these isoforms is regulated during granulocyte maturation. According to functional studies, BID_{EL} induces apoptosis, whereas BID_S abrogates the proapoptotic effects of tBID and inhibits Fas-mediated apoptosis.[238] However, BID_{ES} is also able to partially inhibit the proapoptotic effects of tBID. These three novel, endogenously expressed, BID forms differ in their expression pattern, their cellular localization and their effects upon apoptosis induction, regulating in this way the function of BID and thus influencing the fate of cells.[238]

c. HRK (DP5, Harakiri). Harakiri *(HRK)* is a proapoptotic gene of the *BCL2* family, mapping to chromosome 12q13.1.[239] Its protein product consists of 91 amino acids and displays a minor homology to BCL2 that is restricted to a stretch of 8 amino acids that share high homology with BH3.[240] HRK protein also contains a hydrophobic domain at its C-terminal region, implying its membrane-spanning ability. The protein presents a restricted tissue distribution since it is predominantly detectable in brain, spleen and bone marrow,[240,241] and its expression is triggered by the deprivation of survival factors such as IL-3 or by chemotherapeutic agents such as etoposide,[242] implying that the protein is regulated at the transcriptional level. Induction of *HRK/DP5* expression has been observed in NGF-deprived sympathetic neurons,[241] in apoptosis of cortical neurons after exposure to amyloid β,[243] in apoptosis of cerebellar granule neurons induced by withdrawal of potassium,[244] in spinal neurons of amyotrophic lateral sclerosis patients,[245] in retinal ganglion cells of axotomized retina,[246] in growth factor-deprived hematopoietic cells[247] as well as in blastomeres during embryonic development.[248] These data are consistent with the presence of a regulatory region at the 3′-untranslated region of the gene, responsible for the binding of the transcriptional repressor downstream regulatory element antagonistic modulator (DREAM). It has been demonstrated that the presence of IL-3 provokes the activation of DREAM and subsequently blocks *HRK* transcription, contributing to cell survival.[249] As mentioned before, overexpression of HRK provokes cell death. Since the protein has the ability to interact with antiapoptotic BCL2 and $BCL\text{-}X_L$, it must contribute to apoptosis by inhibiting the protective effect of these proteins. Recent data demonstrate that, upon stimulation, HRK/DP5 is targeted to mitochondria, where

it interacts with p32, a homotrimeric mitochondrial protein inducing cell apoptosis.[250]

d. BCL2L11 (BAM, BIM, BOD). The BCL2-interacting mediator (BIM) human gene maps on chromosome 2q12 or 13. It consists of 6 exons, the third of which is a facultative intron that is spliced out in the mRNAs for isoforms BIM long form (BIM_L), BIM short form (BIM_S), but not for the largest isoform, BIM extra long form (BIM_{EL}). The region that contains the TATA-less promoter and the binding sites for several transcription factors can drive the expression of a reporter gene.[251] The molecule displays a minimal homology to the BCL2 family members that is restricted to 9 amino acid residues of the BH3 domain and the hydrophobic domain of its C-terminus,[252,253] implying its localization at cytoplasmic membranes.

BIM/BOD is a proapoptotic gene of the BCL2 family. The protein, which has been predominantly detected in hematopoietic, neuronal, epithelial and germ cells,[254] plays a major role in hematopoietic cell homeostasis, in prevention of autoimmunity and in embryogenesis.[255,256] Its activity is regulated at the level of both protein expression and post-translational modifications, such as phosphorylation.[255,257-259]

Though the expression of the larger isoform (BIM_L) predominates at the protein level, the smallest protein (BIM) is the most potent[252,253] in terms of apoptotic induction. Six more isoforms of BIM have since been identified and characterised, designated as BIM alpha 1 (BIMα1), alpha (α2), beta 1-4 (β1-4) and generated by alternative splicing.[260] They do not contain a C-terminal hydrophobic region, and only BIMα1 and α2 contain a BH3 domain and are proapoptotic, though less potent than the classical isoforms described above.[260] When these two isoforms were transiently expressed in human cervix carcinoma epithelial (HeLa) cells, they partially localised in mitochondria.[260] In addition, although the classical isoforms were always predominantly and uniformly expressed in tumour cells or transformed cells, expression profiles of BIM isoforms were highly variable among normal human tissues, such as brain, heart and liver, suggesting a tissue-specific transcriptional regulation of BIM.[260] BIMγ, the most novel isoform of BIM, generated by retention of a 126 bp intron of the *BIM* gene, displays a tissue-specific expression pattern distinct from those of the other BIM isoforms.[261] It is localised both in intracellular membranes and cytosol, and it is up-regulated in the majority of the prostate cancer cell lines studied; clonal growth is inhibited, thus promoting apoptosis.[261]

BIM is a component of the microtubule-associated complex. In healthy cells, the protein is bound to the dynein motor complex via the LC8 dynein light chain.[262] When the cell is exposed to a death signal, such as cytokine for lymphocytes[263] or NGF withdrawal for neurons,[264] BIM translocates to the mitochondrial outer membrane.[262] Since it has been found that BIM/BOD interacts with BCL2, BCL-X_L and the virus derived BHRF-1,[253] it is likely that translocation of BIM/BOD to the mitochondrial membrane leads to

inactivation of the antiapoptotic proteins and subsequent release of cytochrome c.[264,265]

BIM_{EL}, the largest alternative splice variant of BIM, is sequestered to microtubules in an unphosphorylated form by means of a direct interaction with tubulin. Its phosphorylation, probably by the ERK1/2 pathway at Ser65,[266] leads to its release from microtubules and its cleavage at the N-terminus by caspases early in the process of apoptosis, induced by stimuli that activate either the mitochondria or the death receptor-dependent apoptotic pathway.[267] The activation of BIM_{EL} downstream from the caspase cascade may provide a positive amplification of apoptotic signals.[267] In addition, its phosphorylation by ERK1/2 at Ser69 results in its degradation by the proteasome pathway,[268] whereas its phosphorylation by c-Jun NH2-terminal kinase (JNK) potentiates BAX-dependent apoptosis induction.[269]

BIM ectopic expression in different cells, including T lymphocytes, induces a drop of mitochondrial membrane potential and apoptosis.[270] BIM promotes apoptosis by binding to and inhibiting antiapoptotic proteins of the BCL2 family.[271,272] In addition, activation of lineage kinases/c-Jun N-terminal kinases,[273–275] as well as inactivation of Ras/mitogen-activated protein kinase,[274] extracellular signal-activated kinases,[276–278] and PI3/Akt signalling pathways[268,279–280] have been shown to account for BIM induction or activation. According to recent data, BIM is up-regulated after T cell receptor triggering in human T cells, implicating BIM as a mediator of death receptor-independent activation-induced cell death.[281]

e. BNIP (NIP) Proteins. BCL2 and the nineteen kDa interacting protein (BNIP) proteins, including homologues isolated from human, mouse and *Caenorhabditis elegans*, are a relatively new subgroup of the BCL2 family. They were first discovered based on their interaction with the adenovirus E1B 19kDa/BCL2 family proteins.[282] They are proapoptotic members of the BCL2 family, playing a central role in the regulation of mitochondrial membrane permeability. The BNIP group of proteins includes: BNIP1, 2, 3 and NIX in humans; mBNIP21, a BNIP2 homologue, in rodents and ceBNIP3 in *Caenorhabditis elegans*.[283,284]

f. BNIP1. BNIP1 protein consists of 228 amino acids that contain a C-terminal TM domain, the presence of which enables the protein to form a stable association with cellular membranes and also accounts for its reported localization to the nuclear envelope and/or endoplasmic reticulum.[282] Three alternatively spliced protein forms of BNIP1 have been isolated, known as BNIP1 a, b, c.[285] Among them, BNIP1 and BNIP1-b contain a BH3 domain, while BNIP1 a and BNIP1-c do not. The absence of the BH3 domain in the a and c variants does not prevent their interactions with BCL2,[285] although in BNIP1 the BH3 domain is important for its apoptotic function.[286] In addition, BNIP1 and its variants show a wide pattern of expression in a number of tissues, including brain, heart, placenta, lung, skeletal muscle and kidney, without their specific roles being fully elucidated.

g. BNIP2 and mBNIP21. BNIP2 is a 315 amino acid protein, which shares 47% identity and 66% similarity to the human Rho GTPase-activating protein (GAP).[282] It does not contain a TM hydrophobic domain, and it localises at the nuclear envelope and the endoplasmic reticulum. Transfection of BNIP2 cDNA showed that BNIP2 could induce massive cell death and that BCL2 overexpression restored cell viability.[287]

Two BNIP2 isoforms were identified, BNIP2-Sα (longer) and BNIP2-Sβ (shorter), which show differential expression patterns in various cells and tissues, with the expression of BNIP2-Sα causing extensive apoptosis in cells.[288] A homologous protein of human BNIP2 was isolated from hearts of coxackievirus B3 (CVB3) infected A/J (H2) mice, known as mBNIP21. The *mBNIP21* gene encodes for a 326 amino acid residue and shares 925 sequence homology with human BNIP2. It is a proapoptotic protein that was found to induce apoptosis in HeLa cells and cultured cardiomyocytes through a mitochondria-mediated pathway, in which BID is cleaved by caspase-8 and tBID induces cytochrome c release from mitochondria, followed by caspase activation.[289]

h. BNIP3. *BNIP3* (BCL2 and the nineteen kDa interacting protein-3) gene, the most well-characterised member of the BNIP group of proteins, resides on chromosome 14q11.2–12,[290] and consists of 657 nucleotides encoding for a protein of 194 amino acids that is widely expressed[291] and is regulated at the transcriptional level. Several homologues of BNIP3, sharing both structural and functional similarities, have been identified in mammals; they are Nix (also called BNIP3L/BNIP3a/B5),[291] BNIPL,[292,293] BNIP3H[294] and in *C. elegans*, ceBNIP3.[283,284] BNIP3 protein (19 kDA interacting protein) was isolated because of its ability to dimerize with the adenovirus-derived E1B 19K protein, provoking cell death and rescuing the organism from inflammation. It is a proapoptotic, mitochondrial, BH3-only domain protein, containing a BH3 motif and a C-terminal TM domain.[295] In yeast and mammalian cells, BNIP3 interacts with the survival-promoting proteins BCL2, BCL-X_L and cell death protein-9 (CED-9). Typically, the BH3 domain of proapoptotic BCL2 homologues mediates BCL2/BCL-X_L heterodimerization and confers proapoptotic activity. The native protein has a molecular weight of 30 kDa and covalently dimerizes with a 60 kDa molecule.[290] However, BNIP3, lacking its BH3 like domain, is still able to heterodimerize with BCL2, BCL X_L and CED-9 and can efficiently induce cell death, with this interaction occurring at the NH2 and COOH termini of the BNIP3 protein.[296]

Overexpression of BNIP3 induces cell death as a consequence of early dilated cardiomyopathy (DCM) and reactive oxygen species (ROS) production which is blocked by cyclosporin A or bongkrekic acid, inhibitors of mitochondrial permeability transition (PT) pore opening.[297] In contrast, BNIP3 overexpression does not induce cytochrome c release, and the broad spectrum caspase inhibitor Ac-zVAD.fmk was ineffective in inhibiting cell death, suggesting that BNIP3-mediated cell death is caspase independent.[297]

i. NIX/BNIP3L. The *Nix* gene maps on chromosome 8p21, and its protein product is a significant homologue to BNIP3, possessing a C-terminal TM domain, a putative BH3 domain and a PEST sequence.[290] Similar to the latter, the protein homodimerizes and heterodimerizes with E1B 19K protein when overexpressed. NIX localises in the mitochondrial membrane, and this localization is essential for its proapoptotic activity, since removal of the TM domain leads to its cytoplasmic distribution and subsequent inactivation.[290] The TM domain is significant for the interaction of NIX with BCL2 or BCL-X_L, the presence of which does not inhibit its apoptotic activity. However, higher levels of these antiapoptotic molecules can block its function.[290] *In vivo,* it is rapidly degraded, and its degradation is blocked by treatment with lactacystin, a proteasome inhibitor.[290]

j. ceBNIP3. The orthologue of BNIP3 in *C. elegans* (ceBNIP3), possesses a 21% amino acid sequence identity with BNIP3. It contains a C-terminal TM domain, conserved domain (CD) of 19 amino acids, a BH3 domain and a PEST sequence. ceBNIP3 is expressed primarily as a 25 kDa monomer and a 50 kDa homodimer.[283,284] After transfection, ceBNIP3 protein is rapidly degraded through an ubiquitin-dependent pathway by the proteasome. Like BNIP3, the TM domain of ceBNIP3 mediates its localization to mitochondria, being also necessary for homodimerization and cell death in mammalian cells. Neither the putative BH3 domain nor the conserved domain is necessary for killing.[284] ceBNIP3 protein interacts with CED-9 and BCL-X_L, but unlike other proapoptotic BCL2 family members, the BH3-like domain does not participate in dimerization. The ceBNIP3 TM domain mediates interaction with both CED-9 and BCL-X_L and interacts with CED-3, but co-expression of CED-3 and ceBNIP3 does not significantly enhance induction of cell death in the presence or absence of CED-4.[283] ceBNIP3 kills mammalian cells by a caspase-independent mechanism. In conclusion, although ceBNIP3 interacts with CED-9 and CED-3, it kills via a BH3- and caspase-independent mechanism.[283]

k. BIK (NBK, BP4, BBC1, BIP1). The *BIK* gene, also known as *NBK,* maps on chromosome 22q13.3 and spans a region of 19 kb consisting of 5 exons and 4 introns. It is characteristic that its 5′ flanking region lacks the typical CAAT and TATA boxes.[298] The encoded protein is a human BH3 only proapoptotic protein, sharing only a region of 9 amino acid residues with BCL2 members[299] displaying high homology and similar functions to the murine protein BLK, and it contains a predicted transmembrane segment at its C-terminus. Northern blot analysis indicates an elevated expression of the gene in heart and skeletal muscle[298] and restricted expression in kidney, pancreas, lung, liver, prostate and testis,[300] whereas its intracellular localisation involves the endoplasmic reticulum in addition to a possible location in mitochondria.[301]

The protein interacts with the antiapoptotic molecules BCL2 and BCL-X_L, and additionally with BHFR1 and the adenovirus-derived E1B-19k.[302,301]

Although the protein heterodimerizes with antiapoptotic molecules, it has been found that this is insufficient to cause death. Mutation analysis revealed that deletion of the BH3 domain affected heterodimerization activity but not proapoptotic function,[304,305] therefore implying that BIK/NBK mediates apoptosis via a mechanism independent of dimerization. This protein may be phosphorylated at Ser35 and Thr33 to display sufficient activity, possibly by a casein kinase II-related enzyme,[305] in order for apoptosis to occur. Furthermore, mutation of the phosphorylation sites reduced its apoptotic activity. However, BIK/NBK expression is also enhanced by extracellular death signals such as seprin CrmA, calcineurin and PI3K.[306,307] BIK also may be rapidly turned over during apoptosis.[308]

l. BLK (MGC10442). Murine BLK protein resides in mitochondrial membranes and interacts with the antiapoptotic molecules BCL2 and BCL-X_L, inducing apoptosis via caspase activation.[306,309] Since its proapoptotic activity is inhibited by mutations in the BH3 domain, by overexpression of BCL2 and BCL-X_L and by reduction of the caspase 9 levels, it is evident that BLK promotes cell death through activation of the cytochrome c/Apaf-1/caspase-9 complex. The presence of BCL2 or BCL-X_L prevents BLK from inducing cytochrome c release,[309] whereas treatment of human B lymphoma Namalwa cell line with camptothecin (CPT) leads to BIK translocation to mitochondria.[310]

m. PMAIP1 (NOXA, APR). The human *NOXA* gene is located on chromosome 18q21, and encodes a 54 amino acid protein product which belongs to the proapoptotic BH3-only branch of the BCL2 protein family, whereas mouse NOXA encodes for a 103 amino acid protein, possessing two (A and B) BH3 motifs and is constitutively expressed in small amounts in the brain, thymus, spleen, lung, kidney and testis of adult mice.[311] Both human and mouse NOXA possess a p53 response element at their promoters.[311]

NOXA is a crucial mediator of p53-dependent apoptosis in the liver[312] induced by X-ray irradiation.[313] Its mechanism of action involves its translocation to mitochondria, leading to cytochrome c release and caspase 9 activation.[311] Two domains of NOXA protein, BH3 and mitochondrial targeting domain, are essential for the release of cytochrome c from mitochondria to the cytosol through activation of the permeability transition-related pore, revealing a pathway of mitochondrial dysfunction distinct from that induced by tBID.[314]

Human NOXA induced apoptosis in various cells, including Saos2 cells, in a BH3 motif-dependent manner. Increased expression of NOXA mRNA was observed in Saos2 cells infected with adenovirus encoding p53[315] as well as in BAF 3 cells induced to undergo apoptosis by irradiation.[315] When an antisense oligonucleotide to NOXA was exposed to both Saos2 and BAF-3 cells, the increased expression of endogenous NOXA in response to p53 was inhibited, as was p-53-induced apoptosis, whereas control oligonucleotide had no effect.[315] In addition, ectopic expression of NOXA in HeLa or other cell

lines with adenovirus-mediated gene expression, independently of their p53 status, caused apoptosis in more than 90% of cells 24 h after virus infection.[314]

n. BMF. BCL2 modifying factor (BMF) is a BH3-only protein that binds to prosurvival BCL2 family members, such as MCL1, BCL2, BCL-X_L, and BCL W, to initiate apoptosis, but does not interact with the tested proapoptotic family members (BAX, BID, BAD).[316] The proapoptotic activity of BMF is regulated by its interaction with the myosin V actin motor complex through its binding to dynein light chain 2 (DLC2), which, in healthy cells, sequesters it away from the sites where antiapoptotic BCL2 family members reside (nuclear, endoplasmic reticulum and mitochondrial membranes).[317] In response to stress stimuli, such as ultraviolet irradiation or detachment of adherent cells from their substratum (anoikis), BMF is released from the myosin V motor complex, translocates and binds to antiapoptotic BCL2 family proteins, such as MCL1, BCL2, BCL-X_L and BCL-W.[316–318] In addition, BMF and BIM are phosphorylated by JNK, causing their release from the motor complex.[319]

BMF mRNA has been detected in B and T lymphoid, myeloid and fibroblast cell lines, as well as in mouse embryos at all developmental stages.[317] BMF protein has been detected in many mouse organs, predominately in liver, kidney, pancreas and hematopoietic tissues.[317] Its expression in human Jurkat T lymphocytes, L929 mouse fibroblasts and IL-3-dependent FDC-P1 mouse promyelocytic cells induced apoptosis in about 80% of Jurkat cells within 24 h and reduced L929 colony formation by 65%, which could be inhibited by caspase inhibitor or by coexpression of BCL2 or its homologues.[317]

Two alternative splice variants of BMF, BMF-II and BMF III, were identified and cloned from B-chronic lymphocytic leukemia (B-CLL) cells cDNA. Both of them lacked the BH3 domain, still retaining their DLC2-binding domain. Expression of BMF in HeLa cells reduced colony formation and induced apoptosis, whereas BMF II and BMF-III expression resulted only in colony increase. Furthermore, the expression profiles of BMF mRNA and those of its alternative splice variants differ, since BMF was expressed in many cell types, but mostly in normal and leukemic B lympoid cells, whereas BMF-II and BMF-III were mainly expressed in B-CLL and normal B cells, although they were downregulated in B-CLL cells by serum deprivation.[320]

o. BBC3 (PUMA/JFY1, JFY1, PUMA). The p53-up-regulated modulator of apoptosis gene (PUMA), also known as *JFY1* or *BBC3* (BCL2 binding component 3), was mapped to chromosomal locus 19q13.3 q13, which is frequently deleted in head/neck squamous cell carcinoma (HNSCC) and lung cancer,[321–327] and encodes a BH3-only member of the BCL2 family.[328] *PUMA* was initially identified as a gene activated by p53 in cells undergoing p53-induced apoptosis[328–330] and as a protein interacting with BCL2.[325] It is characterized by low expression levels, a GC-rich coding sequence, and lack of sequence homology to known apoptotic proteins. As a result of alternative splicing, the gene encodes for two BH3-domain-containing proteins,

PUMA-α and PUMA-β, which show similar activities. They bind to BCL2 and localise to the mitochondria, inducing the release of cytochrome c and activating the apoptotic pathway.

Yu *et al.* determined in 2001 that the 193 amino acid PUMA protein is exclusively mitochondrial, and binds to BCL2 and BCL-X_L through a BH3 domain, inducing apoptosis.[328] Exogenous expression of PUMA in colorectal cancer cells,[328] as well as overexpression of PUMA in various cancer cell lines[328] and induction of endogenous p53 through treatment with DNA damaging agents,[328] all resulted in apoptosis induction regardless of the p53 status of the cell.[331] In particular, apoptosis induction by PUMA overexpression in K562 cells was through a BAX-dependent mitochondrial pathway.[332] PUMA is required for apoptotic pathways induced by multiple and diverse apoptotic stimuli, such as ionising radiation (IR), cytokine withdrawal and deregulated c-Myc expression.[331] Furthermore, *PUMA* mRNA levels were increased in response to distinct apoptotic stimuli, such as thymocyte treatment with dexamethasone or serum deprivation of tumour cells, whereas growth factors, such as IGF-1 and epidermal growth factor (EGF), suppressed *PUMA* expression in serum-starved tumour cells.[325]

D. Newly Identified Genes of the *BCL2* Family (Table 3)

1. *BCLAF1 (BTF, KIAA0164, Bk21119.1*

The BCL2-associated transcription factor (BTF) gene, also known as *KIAA0164*,[333] maps to chromosome 6q22–23, whose deletion is involved in the development of some cancers.[334,335] It encodes a novel protein expressed in a wide variety of tissues such as heart, brain, placenta, lung, kidney, and pancreas, but mainly in skeletal muscle.[333] Two *BTF* transcripts, BTF_S (short) and BTF_L (long), have been identified.[335] Although both appeared to be widely expressed, they were deleted in some tumours. BTF_S differs from BTF_L in just 49 amino acids in the C-terminus region[335] and carries an N terminal rich in glycine and serine, a feature characteristic of transcriptional repressors.[335] Its main function is to bind DNA and act as a death-promoting transcriptional repressor. It has been identified because of its ability to interact with the adenovirus-derived E1B-19K protein.[335] It can also dimerize with the BCL2 and BCL-X_L proteins but not with the proapoptotic protein

TABLE 3 New Members of the *BCL2* Family

Approved gene symbol	Approved gene name	Location	Aliases
BCLAF1	BCL2-associated transcription factor 1	6q22–q23	*KIAA0164, BTF*
BCL2L13	BCL2 like 13 (apoptosis facilitator)	22q11	*MIL1, BCL RAMBO*
BCL2L14	BCL2-like 14 (apoptosis facilitator)	12p13–p12	*BCLG, BCL-G*
MOAP1	Modulator of apoptosis 1	14q32	*MAP-1, PNMA4*
BCL2L12	BCL2 like 12 (proline rich)	19q13.3	
BFK		1p13.1	

BAX, leading to its sequestration in the cytoplasm and abrogating its transcriptional repression activity.

In nonapoptotic HeLa cells, BTF was found throughout the nucleus core, as monitored by indirect immunofluorescence.[336] However, induction of apoptosis by Fas resulted in the relocalization of BTF near the nuclear envelope.[335] In addition, sustained overexpression of BTF in HeLa cells induced apoptosis by a mechanism involving the inhibition of antiapoptotic BCL2 family proteins.[335]

2. BCL2L13 (BCL-RAMBO, MIL)

This widely expressed protein displays a significant homology to the BCL2 family of proteins, containing all four conserved BH domains (BH1, BH2, BH3, BH4), separated from the characteristic hydrophobic C-terminal membrane anchor (MA) by a 250 amino acid insertion with two tandem repeats rich in serine residues.[337] It is characterised by proapoptotic activity and is localised to mitochondria in mammalian cells, although it appears to induce apoptosis independently of the classical mitochondrial signalling pathways.[337] This is consistent with the fact that it does not interact with other pro- or antiapoptotic members of the family or with death receptors.[337] Its proapoptotic capability can be blocked only by inhibitors of apoptosis, known as IAPs, and not by any other antiapoptotic members of the BCL2 family or inhibitors of death-receptor signalling.[337] It is believed that its regions that do not contain BCL2 homology domains and MA domains are likely responsible for the induction of apoptosis, but via an unknown pathway not involving its BH motifs, forming a new subtype of BCL2 family proteins.[337]

A splicing variant, BCL-RAMBO$_\beta$ (beta), was recently cloned from the human lymph node cDNA library.[338] It contains a 98 bp Alu-like sequence, with an exon-like activity, and an in-frame stop codon.[338] This splice variant expresses a premature 104 amino acid protein which contains only the BH4 domain and lacks the C-terminal region.[338] Therefore, it is localised throughout the cytosol without being able to translocate to mitochondria when apoptosis is induced, although it retains its proapoptotic activity after cell treatment with etoposide or taxol.[338] BCL-RAMBO beta is expressed in many adult human tissues, such as lymph node, heart and uterine cervix, although it is absent in human brain tissue.[338]

3. BCL2L14 (BCL-G)

BCL-G is another novel human proapoptotic member of the *BCL2* gene family. The gene maps on chromosome 12p12, consists of 6 exons, and undergoes alternative splicing producing three different proteins designated 'short,' 'median,' and 'long' (BCL-G$_S$, BCL-G$_M$, BCL-G$_L$), whose overexpression in various cell lines, such as COS-7 and HEK293T, induces apoptosis.[339,340] The largest product, BCL-G$_L$ (327 amino acids) is diffusely

distributed in the cytosol and displays a wide tissue distribution, including bone marrow, prostate, pancreas, colon, testis and spleen. It possesses both BH2 and BH3 domains,[339] and it can interact with BCL-X_L, which blocks its proapoptotic function. Alternative splicing at exons 7 to 10 also results in the median-in-size isoform, BCL G_M, whose protein product possesses 276 amino acid residues, lacks the BH2 motif and is expressed mainly in testis (reverse transcription-polymerase chain reaction [RT-PCR] analysis).[340] The smallest splice variant protein, BCL-G_S (252 amino acids), is expressed strictly in testis and localises to cytosolic organelles.[339] It is a BH3 only domain protein and is more potent, in terms of apoptosis induction, than the largest variant.[339] A possible post-translational modification of BCL G protein by ubiquitin-like (Ubi-L) polypeptide acceptor protein might be implicated in murine T cell activation, spleen, thymus and testis because of the complex formed between Ubi-L and BCL G.[341]

4. MAP-1

Modulator of apoptosis-1 (MAP 1) is another proapoptotic BH3 domain-only protein. It can undergo self-dimerization and can also interact with BAX, BCL2 and BCL X_L to form dimers *in vivo* and *in vitro* in mammalian cells. Its association with BAX through its BH3 motif seems to be responsible for its caspase dependent proapoptotic function, which is evident upon overexpression.[342]

5. BCL2L10 (BCL-B, BOO/DIVA)

BCL-B is an antiapoptotic gene[126,343,344] composed of 2 exons and 1 intron.[344] It encodes for a widely expressed protein in adult human tissues (RT-PCR analysis), with its highest levels typically found in liver, pancreas, kidneys, brain and lungs.[343,344] Its closest homologue in amino acid sequence and overall organization is the BOO/DIVA protein in mice[126] and Nr-13 protein in quail.[343] It bears all four conserved BH motifs (BH1, BH2, BH3, BH4), as well as a membrane-targeting hydrophobic domain at its C-terminus.[344] It is capable of interacting either with the antiapoptotic molecules BCL2 and BCL-X_L or with the BH3 domain of the proapoptotic protein BAX, but not BAK, suppressing its death-stimulating activity.[344] It is believed that BCL-B may display differential phenotypes depending on the cell type. Its site of action is close to intracellular organelles, like the nuclear envelope and mitochondria, with which it associates.[344] In addition, its antiapoptotic function is eliminated when its C-terminus is deleted, enhancing the idea that BCL-B mediates apoptosis in a mitochondrial-dependent manner.[344,345] It is mapped on human chromosome 15q21.2.[346] It shares 49% amino acid homology to mouse BOO/DIVA protein, whose gene is localised on chromosome 9 and expressed only in the ovary, and it possesses either anti- or proapoptotic function, depending upon cellular content. However, *BCL2L10* mRNA has been found to be expressed in many human tissues.[346] This discrepancy may be

explained by the PEST sequence, which may have a differential role according to the cell type. PEST is a sequence rich in proline, glutamate, serine and threonine residues, present in the BH4 motif of BCL2L10 protein.[347] Proteins possessing this sequence are very susceptible to proteolysis and usually manifest short intra cellular half-lives.

BCL2L10 interacts with the other regulatory proteins implicated in the apoptotic process, such as calcineurin,[348] Raf-1[349] and CED-4.[350] It shows its antiapoptotic activity by blocking apoptosis in the mitochondrial death pathway induced by withdrawal of IL-3 and BAX expression, by preventing cytochrome c release, caspase-3 activation and mitochondrial transmembrane potential collapse, but not by blockage of apoptosis in the death-receptor pathway. It is believed that its suppressing effect on apoptosis is mainly due to its BH4 motif and its TM domain.[346]

6. *BCL2L12*

We have recently cloned the *BCL2L12* gene, which maps on chromosome 19q13.3–13.4.[351] It consists of 7 coding exons and 6 intervening introns spanning a genomic area of 8.8 kb.[351] One splicing variant missing exon 3 and expressing a 176 amino acid truncated protein with no BH2 homology domain has also been identified.[351] The gene localises at the same chromosome as the genes whose protein products are implicated in malignancies, such as the proapoptotic protein BAX,[149] Ras-related protein R-Ras (RRAS), and interferon regulatory factor 3 (IRF3).[351]

BCL2L12 protein has a predominant molecular mass of 36.8 kDa and contains the conserved BH2 domain of the BCL2 family[351] and a putative BH3 domain. Additionally, it bears repeated PXXP motifs and a proline rich region that is essential for the interaction with the SH3 of tyrosine kinases, such as the protooncogenes *c-Scr* and *c-Abl*.[352,353] It is worth mentioning that it is the first gene identified encoding for a protein that contains both a proline rich region and a BH2 domain. Although the BH3 domain is present in most of the mammalian members of the BCL2 family, another antiapoptotic molecule, BOO/DIVA, also lacks this conserved domain, displaying its activity via a dimerization-independent mechanism. On the other hand, the recent identification of the BAX-binding protein BIF1[150] suggests a probable connecting role of BCL2L12 among the apoptotic proteins and the SH3-bearing oncoproteins.

The classic form of the BCL2L12 protein is highly expressed in the thymus, prostate, fetal liver, mammary, colon, placenta, small intestine, kidney and bone marrow, with lower levels being expressed in all other tissues.[351] The splice variant is highly expressed in fetal liver, spinal cord and skeletal muscle, where it is present at higher levels than the classical form of the gene, compared to the other tissues.[351]

Several putative post-translational modifications have been identified through sequence analysis, such as potential sites for O-glycosylation, phosphorylation by cAMP-dependent protein kinase, protein kinase C and casein kinase 2, as well as several N-myristoylation sites.[351]

7. *BFK*

BFK is a new member of the *BCL2* family of genes, mapped on chromosome 1p13.1 in human and composed of 4 coding exons, both in humans and mice. Its protein product contains BH2 and BH3 conserved domains, but not BH1 and BH4 domains and the C terminal hydrophobic MA region. Research data reveal a closer relation of BFK to BCL-G_L protein structure.[354]

BFK is detected in many tissues, such as stomach, bone marrow, spleen and ovary. Its expression was found to be highly regulated in mammary gland and uterus during pregnancy. Inside the cell, it localises to the cytoplasm, where it does not associate with other organelles.

BFK overexpression leads to weak induction of apoptosis, and it does not bind to any of the known proapoptotic or antiapoptotic protein members of the BCL2 family. Furthermore, it is quite interesting to note the expression of BFK during pregnancy, where its high levels in the mammary gland suggest that it may play a role in the regulation of apoptosis in that organ as well as in its development process.[354]

III. DISEASES RELATED TO APOPTOSIS

In healthy tissues, apoptosis helps the organism to maintain its homeostasis since it is opposed to the process of cell proliferation. It is obvious that abnormalities in the apoptotic mechanism lead to the pathogenesis of various diseases or disorders related to cell population numbers. Inadequate apoptosis results in excessive augmentation of cell numbers and has implications for tumorigenesis and autoimmunity. On the other hand, excessive apoptosis reduces the cell population, and is associated with neurodegenerative disorders such as Alzheimer's and Parkinson's disease and AIDS.

A. Nonmalignant Diseases Triggered by Excessive Apoptosis (Table 4)

A number of neurogenerative diseases, such as Huntington's disease, Alzheimer's disease (AD), Parkinson's disease (PD) and spinal muscular atrophy, are triggered by excessive reduction of specific neuronal populations. AD is a disorder of the central nervous system induced by the formation of the beta amyloid (Abeta) peptides in the brain. This disorder is associated with alterations in the BCL2/BAX ratio in the neurons. Thus, down-regulation of the antiapoptotic protein BCL2 and up-regulation of the proapoptotic BAX is observed.[355,356] BCL-X(L), another antiapoptotic molecule, can inhibit

TABLE 4 BCL2 Family Members and Diseases Triggered by Excessive Apoptosis

Gene symbol	Disease
BCL2	Alzheimer's, Parkinson's, Down Syndrome, AIDS
BAX	Alzheimer's, Parkinson's, AIDS
BCL-X(L)	Alzheimer's
BIM	Alzheimer's, Down Syndrome

the Abeta-induced neuronal apoptosis.[357] Moreover, patients with AD have shown a significant increase in frontal cortex BIM levels, as well as cerebellar BCL2 levels, supporting the apoptotic origin of neuronal loss in AD.[358] BCL2 and BAX are also implicated in Parkinson's disease. This disorder is triggered by the apoptotic death of dopaminergic neurons (DA).[359] In the nigrostriatal dopaminergic regions of Parkinson's patients, the concentration of BCL2 has been observed to be significantly higher than that of the control.[360] In patients with Down syndrome (DS), cerebellar and, to a lesser extent, frontal cortex BIM and BCL2 levels were elevated, supporting the hypothesis that apoptosis accounts for the neuronal loss of DS.[358]

AIDS develops after exposure to the human immunodeficiency virus (HIV). This virus induces apoptosis of the T-helper cells, which are responsible for the survival and the activation of the T-cytotoxic cells. T-cytotoxic cells are implicated in the execution of viruses and cancer cells.[4,361] Thus, the human immunodeficiency virus indirectly blocks the activity of the T-cytotoxic cells, making the organism vulnerable to viral inflammations and secondary malignancies. It has been postulated that the HIV envelope protein provokes reduction of the BCL2 protein and the simultaneous induction of BAX via the Fas/FasL pathway.[362,363] Exogenous addition of IL-2 or Fas ligands leads to the prevention of both BCL2 reduction and apoptosis of T lymphocytes from HIV-infected individuals, whereas no correlation was evident between BAX or BCL-X expression and apoptosis induction.[362–364]

B. Nonmalignant Diseases Triggered by Reduced Apoptosis (Table 5)

Decreased apoptosis of autoreactive immune cells, in addition to their induction and proliferation, plays an important role in the development of autoimmunity. In lymphocytes, Fas is inductively expressed as a response to

TABLE 5 *BCL2* Family Members and Diseases Triggered by Reduced Apoptosis

Gene symbol	Disease
BCL2	Systemic lupus erythematosus, Hashimoto thyroiditis
BIM	Glomerulonephritis
BNIP3	Cardiovascular diseases
BCL-X	Non-obese diabetes
BTF	Emery-Dreifuss muscular dystrophy

FasL, which is only produced in activated T cells. Self-reactive lymphoid cells are induced to undergo apoptosis when their Fas receptors bind FasL.[365]

BCL2, BAX, BIM and the surface receptor Fas are usually implicated in autoimmunity. An Ala/Thr polymorphism in BCL2 results in different anti apoptotic function of the protein and is involved in the development of autoimmune diseases.[366] Systemic lupus erythematosus (SLE) is a systemic autoimmune disease in which decreased apoptosis of lymphocytes is observed. In SLE patients, a high proportion of T cells display an increased amount of BCL2, and therefore they are resistant to death stimuli.

BNIP protein involvement in cardiovascular diseases may not be surprising considering the relatively high proportion of mitochondria to fulfill the energy requirement of cardiac contraction. Expression analysis of BNIP3 in various human tissues showed high expression in the heart, brain and testis. Increased expression of BNIP3 was found in hypoxic cardiomyocytes,[367] and subsequent acidosis could induce BNIP3 activation, determined by its integration into the mitochondrial membrane and the induction of cardiomyocyte cell death.[368] Thus, BNIP3 may have important roles in ischemia-reperfusion injury of the heart. Overexpression of BNIP3 itself in normoxic cultured cardiomyocytes is sufficient to induce apoptotic cell death in a caspase-dependent manner.[367]

In Hashimoto thyroiditis, a lack in *BCL2* expression is observed with a simultaneous up-regulation of FasL, leading to excessive death of the thyroid cells.[369–371] In autoimmune non-obese diabetic (NOD) mice, up-regulation of the *BCL-X* gene results in resistance to apoptosis of the activated lymphocytes after withdrawal of IL-2.[372]

BIM is associated with autoimmune kidney disease. It has been observed that *BIM* deficiency in mice provokes the accumulation of lymphoid, plasma and myeloid cells.[255,265] This accumulation leads to the fatal autoimmune lymphoproliferative disease, glomerulonephritis,[265] in these animals due to impaired apoptosis.

Using a two hybrid system in HeLa cells, it was found that emerin binds to BTF. Such an interaction is disrupted by the disease-associated S54F missense mutation in emerin, possibly resulting in EDMD (Emery-Dreifuss muscular dystrophy) disease.[336]

C. Apoptosis and Cancer (Table 6)

The importance of apoptosis inhibition during the process of tumorigenesis has been recognized, and the ongoing discovery of numerous apoptosis-regulating proteins provides new potential targets for molecular cancer therapy. Among these are the members of the BCL2 family.

Deregulation of Bcl2 family members has been tightly linked to tumorigenesis.[50] All antiapoptotic Bcl2 homologues seem to function as oncoproteins, and proapoptotic BH3-only and BAX-like proteins can act as

TABLE 6 BCL2 Family Members and Cancer

Gene symbol	Cancer type
BCL2	Non-Hodgkin's follicular B-cell lymphoma, prostate cancer, colon cancer, lung cancer, breast cancer, gastric cancer, renal cancer, neuroblastoma, acute and chronic leukemia, skin cancer
BCL-X	Lymphoma, colon cancer, pancreatic cancer, hepatocellular cancer, renal cancer, breast cancer, ovarian cancer, acute T lymphocytic leukemia
BAD	Colorectal cancer, B cell lymphoma
BID	Chronic myelomonocytic leukemia
BAX	T-cell lymphoma, breast cancer, lung cancer, lymphoblastic leukemia, gastrointestinal cancer, glioblastoma multiform
BAK1	Colorectal carcinoma, gastric cancer, skin cancer, uterine cervix cancer, thyroid cancer, lung cancer, lymphoid cancer, breast cancer, glioblastoma multiform tumours
BCL2L2	Colorectal adenocarcinoma, infiltrative gastric adenocarcinoma
NOXA	Urinary bladder cancer
PUMA	Head/neck cancer, lung cancer, colorectal cancer
BTF	Bladder cancer
BIM	B cell leukemia
BCL-G	Prostate cancer
BCL2L10	Cervical cancer, ovarian cancer, leukemia
BCL2A1	Bladder cancer, B-cell neoplasia, breast cancer
BCL2L12	Breast cancer, colon cancer, leukemia
BIK	Breast cancer, colorectal cancer
MCL1	Multiple myeloma, prostate cancer, B-cell chronic lymphocytic leukemia, plasma cell myeloma, leukemic relapse in AML and ALL, colorectal cancer, malignant myeloma, ovarian carcinoma, hepatocellular carcinoma, breast cancer

tumour suppressors. This is supported by a comparative genomic hybridization (CGH) database search for copy number gains and/or deletions of chromosomal loci from members of the *BCL2* family that indicates a specific role of these genes and their pro- and antiapoptotic products during the pathogenesis of tumours.

1. BCL2. The *BCL2* gene was first discovered because of its t(14;18) chromosomal translocation, detected by PCR, in non-Hodgkin's follicular B-cell lymphomas[23,24] as well as in chronic lymphocytic leukemia with less frequency.[373] This leads to its transcriptional activation and, thus, overproduction of BCL2 protein in B cells, which contributes to tumorigenesis by preventing cell death rather than by causing cell proliferation.[28]

Overexpression of *BCL2*, detected mainly by immunohistochemical methods, takes place in distinct types of human tumours, including those of prostate, colon, lung, breast, gastric cancers, renal cancers, neuroblastoma, non Hodgkin's lymphoma, acute and chronic leukemia and skin cancers.[374–386] In some of these cancer types, its up-regulation can be used as a prognostic marker,[280,384,387] such as in breast cancer where *BCL2* expression has been associated with a favorable prognosis.[42,388] However, in other types, it does not correlate with disease progression.[389–391] In most cases,

overexpression of the *BCL2* gene is associated with resistance to chemotherapeutic and irradiation treatment.[388] For example, high levels of the BCL2 protein in acute myeloid leukemia (AML) cells indicate a poor response to chemotherapeutic agents in terms of remission outcome and survival.[389,390] *BCL2* overexpression in B cell lymphomas results in poor patient outcome.[391] Higher *BCL2* expression was detected in AML types M0 and M1 where a correlation was detected with resistance to chemotherapy and poor prognosis as well as decreased rates of complete remission and shortened survival.[392] However, overexpression of *BCL2* failed to inhibit apoptosis induced by CD437 in HL-60 cells.[393]

In lymphoma cells, the apoptotic function of the BCL2 protein and, therefore, its involvement in carcinogenesis is partly regulated by phosphorylation/dephosphorylation of the protein.[394] Phosphorylation of BCL2 at Ser70 promotes its antiapoptotic function, whereas its multisite phosphorylation observed after treatment of cells with microtubule-damaging agents, such as taxol, inhibited its antiapoptotic function. Treatment of AML cells with taxol led to the production of different phosphorylation levels of BCL2, caused by distinct signalling pathways.[395]

Many chemotherapeutic drugs have been designed to fight against tumour progression by down-regulating the production of the BCL2 protein in tumour cells. Okadaic acid, used to treat leukemia,[396] and retinoic acid treatment of human acute promyelocytic leukaemic cells, provoke the down-regulation of the *BCL2* gene and improved response to chemotherapy. However, pretreatment of myeloid leukemia cells with dexamethasone led to BCL2 down-regulation but did not enhance cell sensitization to apoptosis. Furthermore, lower *BCL2* expression has been correlated with poorer clinical outcome in patients with metastatic breast carcinoma. In addition, treatment of cells with BCL2 antisense oligonucleotides, or small cell-permeable molecules that bind to and inactivate BCL2, sensitized cells to apoptosis induction.[397–401]

2. *BCL-X.* BCL-X is another protein that is variously involved in cancers. The identification of a new promoter and a new exon-1 in the gene can explain the cancerous potential of the protein. This new exon 1, the so-called exon IB, replaces the untranslated IA exon of the wild-type gene while the new promoter, which is found upstream of the exon IB, is 300-fold more potent.

BCL-X protein, as detected by immunohistochemical methods, is widely expressed in a variety of human malignancies, including lymphoma, colon, pancreatic, hepatocellular, renal, breast and ovarian cancers.[402–404] In some types of cancer, the reduction of the protein concentration can also take place at the transcriptional level. Human acute T lymphocytic leukemia (ATLL) is developed after infection by the human T lymphotropic virus types I (HTLV-I) and II (HTLV-II). The Tax1 and Tax2 transactivators of HTLV-I and II increase the transcription of the $BCL\text{-}X_L$ gene and, therefore, are responsible

for the development of the disease.[405] The down-regulation of the *BCL-X_L* gene is in close relationship with c-myb expression and represents a useful prognostic marker in colorectal carcinoma.[406] In breast carcinoma cells, the excessive concentrations of BCL-X_L and BCL2 inhibits the protective action of TNF.[407]

3. BAD. In colorectal and other types of cancer,[408] lack of BAD/BCL2 heterodimerization occurs due to BAD hyperphosphorylation, as has been observed in a variety of malignant cell lines.[409] Since the phosphorylated form of the protein is sequestered in cytosol as a complex with the 14-3-3 protein,[410] it is unable to prevent tissues from excessive growth.

Mutation analysis in colon adenocarcinomas showed two somatic missense mutations in the *BAD* gene, both of which were located within the gene sequence encoding the BCL2 homology-3 domain. Such mutants had decreased apoptotic activity compared to the wild-type BAD as well as reduced binding ability for BCL2 and BCL-XL. Ranger *et al.*[411] observed that *BAD*-deficient mice spontaneously developed tumours, which were mainly B cell lymphomas.

4. BID (MGC15319, MGC42355) BID-deficient mice, as they grow older, spontaneously develop a myeloproliferative disorder that progresses from myeloid hyperplasia to a fatal, clonal malignancy, closely resembling chronic myelomonocytic leukemia (CMML).[412] This apoptotic defect can result in myeloid leukemogenesis. Premalignant *BID-/-* myeloid precursor cells are resistant to death receptor-induced apoptosis. Cleavage of BID by an undefined aspartate-specific protease can also be a key mediator of the apoptotic response to several DNA-damaging anticancer regimens.[413–415]

5. BAX (Bax zeta) BAX is a tumour suppressor, which means that, in healthy cells, its function provokes the apoptotic death of excessive or damaged cells, contributing to tissue homeostasis. However, in malignant incidents, the concentration of this protein in cancer cells is reduced. *p53*-deficient mice present reduced BAX levels and develop T cell lymphoma.[416] In most cases of cancer, the reduced concentrations of BAX are accompanied by mutations in the *p53* gene. A missense mutation in codon 273 of the *p53* gene can dramatically decrease the BAX levels in the cell.[417] In breast cancer, mutational analysis of the *BAX* and *p53* genes identified mutations of the *p53* gene but no mutations of the *BAX* gene, except for a G → A polymorphism at exon 6 position 552 in all individuals tested.[418] In lung cancers, BAX is localised in the nucleus, and this position-translocation is considered to cause tumour development. However, mutational analysis of the gene in lung cancers revealed the presence of only a silent point mutation in codon 184 (TCG → TCA) and some intronic mutations that do not affect the TM domain of the protein.[419]

Frameshift mutations have been detected in the *BAX* gene in T cells in acute lymphoblastic leukemia[420] and endometrium.[421] In all cases of gastrointestinal cancers, there are two characteristic missense mutations of

BAX gene in codon 169 (Thr → Ala or Thr → Met). These mutations are functional, and, therefore, they inhibit the proapoptotic activity of the protein and contribute to carcinogenesis.[122]

On the other hand, a splicing variant of *BAX* gene, BAX-sigma, lacking amino acids 159 to 171 compared to BAX-alpha, is widely expressed in human cancer promyelocytic cells and in a variety of other human cancer cell lines.[152] It has recently been shown that the expression of another apoptogenic variant of BAX, BAX-Ψ, was associated with a longer survival of glioblastoma multiform (GBM) patients,[6] an especially severe type of tumour.[18] BAX-Ψ was found to be expressed in 25% of the patients examined, while very few tumours lacked the expression of BAX.

Using CWR22 xenografts, an interaction of PKCε was identified with the proapoptotic protein BAX that was unique to recurrent CWR22 tumours.[423] These data suggest that an association of PKCε with BAX may neutralize apoptotic signals propagated through a mitochondrial death-signalling pathway.[423]

Various chemotherapeutic treatments aim at up-regulation of the *BAX* gene to block tumour progression. For example, all the anthracycline group chemotherapeutic agents fight cancer through enhancement of BAX expression.[424] The potent tumour-suppressing activity of this protein is now used for therapeutic purposes. Transfected adenoviral systems that overexpress BAX protein are used to infect the target tumours in many types of cancer, including prostate and ovarian cancers and gliomas.[425–427]

Recently, a C2-ceramide-resistant HL-60 subline, HL-CR, was established.[128] These cells were resistant not only to ceramide but also to other anti-cancer drugs, including daunorubicin, etoposide and cytosine arabinoside. It was found that *BAX* was highly expressed in HL-60 but was hardly detected in HL-CR cells. Transient transfection with BAX-expressing plasmids induced apoptosis in HL-CR cells, suggesting that reduced expression of *BAX* may play a role in resistance to various apoptosis-inducing stimuli in these cells.[428]

6. BAK1 (BCL2L7, BAK, CDN1) BAK is another protein whose expression, as assayed by immunohistochemistry, is modulated in cancerous conditions. In colorectal carcinomas, the increased levels of BAK/BCL2 heterodimerization contribute to tumour growth.[408] In normal gastrointestinal epithelial tissues, up-regulation of BAK expression during differentiation may help to ensure that cell turnover occurs in a normal fashion, whereas gastric tumours have reduced BAK levels when compared with the normal mucosa.[429] This down regulation of BAK may be explained by the presence of missense mutations in the *BAK* gene.[430]

Proteolytic degradation of BAK is also associated with some other types of malignancies. Indeed, in skin cancer, BAK is proteolytically degraded by the human papillovirus (HPV) protein E6.[431] Thus the protein is unable to respond to UV killing of the damaged cells. In addition, adenoviral-mediated

overexpression of BAK can be used as treatment for the targeted malignant cells.[184,432]

The *BAK* gene has been mapped to chromosome 6 p 21.3,[433] where a high incidence of loss of heterozygosity was found in cancer of the human uterine cervix.[434] Since transformation of the cervical epithelial tissue to carcinoma is associated with the progressive inhibition of apoptosis[435] and BAK-mediated apoptosis in these cells,[436] abrogation of this BAK-mediated apoptosis could lead to transformation of the cervical epithelium. The *BAK* gene may therefore act as a tumour suppressor in cancer of the uterine cervix.

Six missense and one silent mutations were recently found, by PCR amplification followed by single-strand conformation polymorphism (SSCP) analysis and direct sequencing, at exons 3, 4 and 6 of the *BAK* gene in carcinomas of the uterine cervix, while no mutations were detected in non-neoplastic cervical tissues.[437] *BAK* mutations were observed more frequently in the advanced stage, at which mutated cancer tissues were even more resistant to radiotherapy.[437]

Further data, based on cDNA microarray analysis, demonstrate that *BAK* is up-regulated in IFNγ-sensitized, TRAIL-mediated apoptosis *in vitro* and *in vivo* in human thyroid cancer cells. In contrast, normal human thyroid cells were not responsive to the IFNγ sensitizing effect, and this was correlated to no evident alterations in the concentration of BAK.[438]

Gene transfer-mediating elevations in BAK protein levels were shown to accelerate apoptosis induced by growth factor deprivation in murine lymphoid, lung cancer and breast cancer cells,[113] suggesting that BAK functions primarily as a promoter of apoptosis. In addition, the expression of BAK was analyzed in human GBM tumours, and it was shown that almost all tumours expressed BAK.[439]

7. BCL2L2 (BCLW, BCL-W, KIAA0271) *BCL-W* is expressed, as assayed mainly by immunohistochemical methods, in colorectal adenocarcinomas,[440] with its expression being modulated by the protooncogene *c-met*.[123] It has been suggested that BCL-W may be implicated in the progression from adenoma to adenocarcinoma in the colorectal epithelium, by inhibiting apoptosis.[440] BCL-W is also expressed in most cases of infiltrative gastric adenocarcinoma, and it may suppress tumour cell death by blocking JNK activation.[441] Its absence in small intestinal epithelium (*BCL-W*-null animals) resulted in a 6-fold increase in the levels of apoptosis induced by 5-fluorouracil or gamma irradiation.[442] In addition, *BCL-W* is expressed both in multiple myeloma and normal plasma cells.[443]

8. NOXA. Mutation analysis of the entire coding region and of all the spliced sites of the *NOXA* gene in a series of human cancers, including those of stomach, colon, liver, urinary bladder and lung, by PCR, SSCP and DNA sequencing, resulted in the identification of one somatic mutation in a transitional cell carcinoma (TCC) of the urinary bladder.[444] Overexpression

of the mutant and wild-type *NOXA* in 293T and HeLa cells had no effect on cell death.[111] These results suggest that *NOXA* is rarely mutated in human carcinomas, and its role in the pathogenesis of human cancer remains unclear. *NOXA* mRNA expression, detected by real-time PCR, was found in tumours from patients with colorectal adenocarcinoma and the corresponding normal mucosa. The expression was up- or down-regulated in a fraction of the cases, but the changes observed did not have any clinical or pathological significance.[445]

9. PUMA Primary tumours from head/neck and lung were analyzed for loss of heterozygosity (LOH) at 19q using seven widely spaced microsatellite markers. LOH was present in 56% of the head/neck and 26.6% of the lung cancer samples, with D19S408 and D19S412 showing the highest rates of allelic loss (23.3 and 16.6%, respectively). However, no mutations of *PUMA* were detected in any samples examined, regardless of the mutational status of the *p53* gene. In colorectal cancer cells, *PUMA* exogenous expression was thought to promote rapid apoptosis,[328,446] but, according to recent data, its expression pattern in colorectal cancer does not differ significantly from normal mucosa when assayed by real-time RT-PCR.[447]

10. BTF BTF gene, also known as *KIAA0164*, is thought to play a role in tumour suppression.[335] In bladder cancer, BTF protein, as monitored by chemiluminescence immunoassays, appeared to be an unfavorable prognostic marker because of its elevated expression in patients who showed recurrence.[448]

11. BIM. Loss of *BIM* in mice suffering from polycystic kidney disease, after loss of *BCL2*, led to normal kidney development, growth and health.[449] On the other hand, loss of *BIM* in normal aging mice caused accumulation of excess lymphoid and myeloid cells.[255] Loss of *BIM* is highly oncogenic. Inactivation of even one of its alleles accelerated MYC-induced development of tumours and, in particular, acute B cell leukemia.[450] It is also proapoptotic since its protein levels were elevated in apoptosis-prone B lymphoid cells.[450] All these data indicate that BIM is a tumour suppressor, at least in B lymphocytes, but it is haploinsufficient.[450]

Treatment of human glioblastoma cells (U87, U251, U138) with lovastatin, a potent cholesterol-lowering agent, led to up-regulation of BIM levels along with the induction of apoptosis,[451] suggesting that it may also have some anticancer activity.

12. BCL-G BCL-G showed significantly higher expression in patients with prostate cancer who relapsed, in comparison to those who did not relapse. This observation was independent of stage and grade and it was estimated by real-time RT-PCR analysis and the statistical Mann-Whitney U test.[452]

13 BCL2L10 (BCL-B, BOO/DIVA) BCL B is frequently expressed in human tumour cell lines[315] and is localised on chromosome 15q21, a locus that shows deletions in human cervical cancer, as shown by PCR and comparative

genome analysis.[126] In addition, its overexpression promoted cell death in HeLa and 292 cells.[126] Recently, alterations in BCL2L10 gene copy numbers, detected by subtractive comparative genomic hybridization studies in the Ara-C resistant cancer cell line K562/AC, in comparison to its drug-sensitive parental cell line, suggested a mechanism for acquired chemoresistance.[453]

14. BCL2A1 (BFL1, BCL2L5, GRS, HBPA1, BCL2A1) BFL1 is up-regulated in cisplatin-resistant human bladder cancer cell lines, which suggests that its differential expression may be important in the cisplatin resistance of human bladder tumour cells.[454] It is also up-regulated when immature B cells are recruited into the long-lived mature B cell pool, preventing antigen receptor binding-induced cell death in B cell lines and mature B cells.[455] Its expression is also a key factor in B-cell neoplasias.[455] Engagement of surface IgM elicits a survival programme in chronic lymphocytic leukemia B cells that is associated with up-regulation of BFL1/A1. In mantle cell lymphomas, inhibition of the constitutively activated NF-κB pathway leads to tumour cell apoptosis in association with down-regulation of *BFL1/A1* expression. In addition, induction of BFL1/A1 was recently found to be essential for breakpoint cluster region/Abelson leukemia (BCR/ABL)-dependent leukemogenesis. Constitutive expression of BFL1/A1 in myeloma cells rescued them from apoptosis induced by IL-6 withdrawal and dexamethasone. According to recent data, spontaneous apoptosis of B-CLL cells in *in vitro* culture resulted in decreased *BFL1* mRNA expression, as assayed by RT-PCR.[456]

The gene is also up-regulated by the Epstein-Barr virus LMP-1 and promotes the proliferation of lymphoma cells.[457] Furthermore, the protein co-operates with *E1a* oncogene in epithelial cells, causing carcinogenesis.[458] In breast cancer, increased expression of *BFL1* was present, being higher in advanced than in early breast cancer and possessing no relationship to other clinicopathological parameters.[459]

Up-regulation of both BFL1/A1 and serine/threonine kinase Pim-1 is essential for *in vitro* transformation and *in vivo* leukemogenesis mediated by BCR/ABL kinases. The *A1* gene cooperates with the *EIA* oncogene in the transformation of primary rodent epithelial cells and has also been reported to be overexpressed in human stomach cancer tissues.[131,134] All the above data reveal the involvement of *BFL1/A1* in carcinogenesis and, more specifically, in stomach cancer development,[131] hemopoietic malignancies and melanoma.[128]

15. BCL2L12 The expression of the *BCL2L12* gene has been analyzed in breast and colon cancer by RT-PCR. Results indicate that *BCL2L12* gene expression may be regarded as a new independent favourable prognostic marker for breast cancer since it is overexpressed more often in breast tumours with a high degree of differentiation as well as in patients in the initial stages of the disease.[460] In colon, both transcripts of the gene (*BCL2L12* and *BCL2L12-A*) were overexpressed in cancer tissues as compared to their

paired normal mucosa. An association was also found between the *BCL2L12* A transcript expression and nodal status as well as with Dukes' stage.[461]

BCL2L12 expression was studied by RT-PCR during apoptosis induced by widely used chemotherapeutic drugs, such as cisplatin, carboplatin and doxorubicin in HL 60 human acute promyelocytic leukemia cells.[462,463] The results revealed important modulations of *BCL2L12* mRNA levels, depending on both the apoptotic inducer and the specific pathway induced, implying a direct relation between alterations of *BCL2L12* mRNA levels and apoptosis.[462,463]

16. BIK (BCL2 Interacting Killer)/NBK Loss of *BIK* is implicated in the development of human breast and colorectal cancers because of the loss of informative alleles on chromosome 22q, where the *BIK* gene is located in many of these tumours.[464] Moreover, BIK can sensitize tumour cells to apoptosis induced by certain chemotherapeutic agents,[465,466] suggesting its implication as a potential therapeutic target for human cancer. The *BIK* gene, when complexed with a non-viral gene delivery system, significantly inhibited growth and metastasis of human breast cancer cells implanted in nude mice, and prolonged the life span of the treated animals.[466] In addition, *BIK* mutants at phosphorylation sites seem to be more potent in their apoptotic and antitumour activity.[467]

17. MCL1 (TM, EAT, MGC1839) MCL1 was initially isolated from MCL1 cells, a myeloid leukemia cell line, with its expression being necessary for the survival of multiple myeloma cells.[81] Elevated levels of MCL1 have also been reported for prostate cancers, B-CLL, plasma cell myeloma[468] and leukemic relapse in AML and acute lymphocytic leukemia (ALL). MCL1 expression has also been correlated with clinical outcome in colorectal cancer patients with liver metastasis; 35% of patients with diffuse MCL1 expression and 90% of patients with peri-nuclear MCL1 expression were found to respond to 5-FU treatment.[469]

Increased *MCL1* expression levels were also observed in primary malignant melanoma (MM), probably representing an early event in MM transformation.[470] Elevated *MCL1* expression was reported for ovarian carcinomas as well, indicating a poor prognostic marker.[471] In hepatocellular carcinoma cells (HCCT), treatment with sodium butyrate causes reversion of the cells to their normal phenotype and affects both differentiation and apoptosis, at the same time up-regulating *MCL1* expression levels.[472] In hydatidiform mole (HM), an abnormal pregnancy with the predisposition for developing gestational trophoblastic neoplasia (GTN), *MCL1* expression levels have been found to be elevated in comparison to HMs that relapsed.[473] In breast cancer, both wild-type MCL1 and $MCL1_S$, its shorter splice variant, were detected.[474] Multiple myeloma was also correlated with increased MCL1 levels in comparison to those in normal plasma cells.[475]

A cyclin-dependent kinase inhibitor, flavopiridol, is currently being tested in clinical trials for cancer therapy. Treatment with flavopiridol not only

arrests the cell cycle but also induces apoptosis through up-regulation of E2F1 and repression of *MCL1*.[476]

IV. CONCLUSIONS AND FUTURE DIRECTIONS

Although cancer is the second leading cause of mortality in developed countries, we do not yet fully understand its pathogenesis. Experience gained from many years of research, has shown that among the best strategies for confronting this disease are early diagnosis through population screening programs, prediction of therapeutic response, and initiation of effective therapy (Figure 6). These conclusions emphasize the requirement for new biomarkers that can be used either alone or in combination with established biomarkers for early disease diagnosis, prediction of response to therapy, and detection of early relapsing disease. These strategies, in combination with the discovery of more effective treatments, may bring a dramatic reduction of morbidity and mortality from all forms of cancer. This, in conjunction with the fact that the human genome project has been completed, leads to a new era for cancer research, in which many new genes related to cancer development or prevention will be discovered and used as cancer biomarkers.

In the last few years, an increasing number of genes involved in cancer development and progression have been cloned and identified, such as *BCL2* family members, kallikreins (*KLKs*), cathepsins and many more.[477–484] Their expression profiles in many types of cancer have been examined, leading to the speculation that many of them may be of high prognostic and diagnostic value for several cancer types, requiring further research and clinical evaluation.

The BCL2 family of proteins (including BCL2L12) plays an important role in moderating the cellular program of apoptosis. Normal cellular homeostasis appears to be dependent on a balance between the actions of

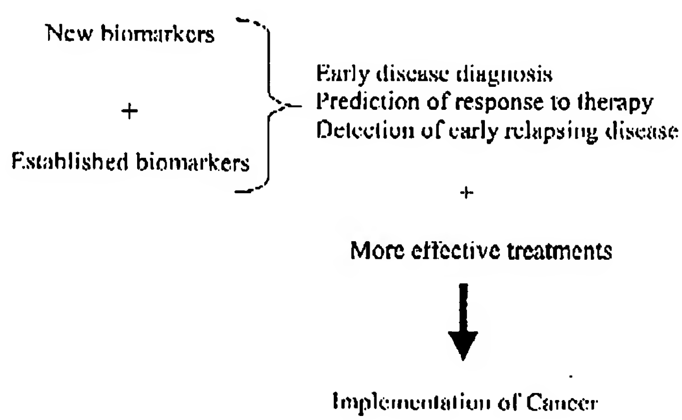

FIGURE 6 Strategies for confronting cancer

antiapoptotic members of the BCL2 family and those of proapoptotic members. The levels of the various members of the BCL2 family have been shown to have prognostic potential and to determine response to chemotherapy in breast tumours and other types of cancer. Results have made it clear that a number of coordinating alterations in the *BCL2* family of genes must occur to provoke carcinogenesis in a wide variety of cancers. However, more research is needed to increase our understanding of the extent to which and the mechanisms by which BCL2 family members are involved in cancer development, providing the basis for earlier and more accurate cancer diagnosis, prognosis, and therapeutic intervention that targets the apoptosis pathway. In addition, new possible markers of the apoptotic process may appear, along with new molecular markers of cancer, that will allow for more effective evaluation of cancer transformation and a corresponding improvement in chemotherapy.

ACKNOWLEDGMENTS

This work was supported by a "EPEAEK II, PYTHAGORAS I" grant (032/319/501104) from the European Community and the Greek Ministry of Education. We thank E. Eleftheriou and E. Katsari for their valuable assistance.

REFERENCES

[1] Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239–257.

[2] Saikumar P, Dong Z, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA. Apoptosis: definition, mechanisms, and relevance to disease. *Am J Med* 1999; 107: 489–506.

[3] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; 267: 1456–1462.

[4] Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med* 1998; 188. 2199–2204.

[5] Hueber AO, Evan GI. Traps to catch unwary oncogenes. *Trends Genet* 1998; 14: 364–367.

[6] Harris AL. Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002; 2: 38–47

[7] Winoto A. Cell death in the regulation of immune responses. *Curr Opin Immunol* 1997; 9: 365–370.

[8] Yin XM, Oltvai ZN, Korsmeyer SJ. BH1 and BH2 domains of Bcl2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* 1994; 369: 321–323.

[9] Cheng EH, Levine B, Boise LH, Thompson CB, Hardwick JM. Bax-independent inhibition of apoptosis by Bcl-XL. *Nature* 1996, 379: 554–556.

[10] Adams JM, Cory S. The Bcl2 protein family: arbiters of cell survival. *Science* 1998; 281: 1322–1326.

[11] Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999, 13: 1899–1911.

[12] Oltvai ZN, Korsmeyer SJ. Checkpoints of dueling dimers foil death wishes. *Cell* 1994; 79: 189–192.

[13] Nguyen M, Millar DG, Yong VW, Korsmeyer SJ, Shore GC. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. *J Biol Chem* 1993; 268: 25265–25268.

[14] Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; 348: 334–336.

[15] Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 1993; 53: 4701–4714.

[16] De Jong D, Prins FA, Mason DY, Reed JC, van Ommen GB, Kluin PM. Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. *Cancer Res* 1994; 54: 256–260.

[17] Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; 275: 1132–1136.

[18] Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997; 275: 1129–1132.

[19] Ottilie S, Diaz JL, Horne W, Chang J, Wang Y, Wilson G, Chang S, Weeks S, Fritz LC, Oltersdorf T. Dimerization properties of human BAD. Identification of a BH-3 domain and analysis of its binding to mutant BCL-2 and BCL-XL proteins. *J Biol Chem* 1997; 272: 30866–30872.

[20] Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem* 1999, 274: 1156–1163.

[21] McDonnell JM, Fushman D, Milliman CL, Korsmeyer SJ, Cowburn D. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. *Cell* 1999, 96: 625–634.

[22] Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* 1996; 381: 335–341.

[23] Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* 1984, 226: 1097–1099.

[24] Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 1985; 228: 1440–1443.

[25] Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 1985, 41: 899–906.

[26] Tsujimoto Y, Croce CM. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci USA* 1986; 83: 5214–5218.

[27] Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 1986; 47: 19–28.

[28] Katsumata M, Siegel RM, Louie DC, Miyashita T, Tsujimoto Y, Nowell PC, Greene MI, Reed JC. Differential effects of Bcl-2 on T and B cells in transgenic mice. *Proc Natl Acad Sci USA* 1992; 89: 11376–11380.

[29] Haldar S, Jena N, DuBois GC, Takayama S, Reed JC, Fu SS, Croce CM. Purification and characterization of the bcl-2 protein. *Arch Biochem Biophys* 1994; 315: 483–488.

[30] Lithgow T, van Driel R, Bertram JF, Strasser A. The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth Differ* 1994; 5: 411–417.

[31] Riparbelli MG, Callaini G, Tripodi SA, Cintorino M, Tosi P, Dallai R. Localization of the Bcl-2 protein to the outer mitochondrial membrane by electron microscopy. *Exp Cell Res* 1995; 221: 363–369.

[32] Hanada M, Aime-Sempe C, Sato T, Reed JC. Structure-function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. *J Biol Chem* 1995; 270: 11962–11969.

[33] Kirkin V, Joos S, Zornig M. The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Acta* 2004, 1644: B229–B249.

[34] Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM, Korsmeyer SJ. Bcl-2 maintains B cell memory. *Nature* 1991; 353: 71–73.

[35] Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S, Harris AW. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc Natl Acad Sci USA* 1991; 88: 8661–8665.

[36] Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ. bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* 1994; 120: 301–311.

[37] Linette GP, Crosby MJ, Hedrick SM, Hansen TH, Glimcher LH, Korsmeyer SJ. Bcl-2 is upregulated at the CD4+ CD8+ stage during positive selection and promotes thymocyte differentiation at several control points. *Immunity* 1994; 1: 197–205.

[38] LeBrun DP, Warnke RA, Cleary ML. Expression of bcl 2 in fetal tissues suggests a role in morphogenesis. *Am J Pathol* 1993; 142: 743–753.

[39] Novack DV, Korsmeyer SJ. Bcl 2 protein expression during murine development. *Am J Pathol* 1994; 145: 61–73.

[40] Michaelidis TM, Sendtner M, Cooper JD, Airaksinen MS, Holtmann B, Meyer M, Thoenen H. Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development. *Neuron* 1996; 17: 75–89.

[41] Murasawa M, Okada S, Obata S, Hatano M, Moriya H, Tokuhisa T. GL7 defines the cycling stage of pre B cells in murine bone marrow. *Eur J Immunol* 2002; 32: 291–298.

[42] Schorr K, Li M, Krajewski S, Reed JC, Furth PA. Bcl-2 gene family and related proteins in mammary gland involution and breast cancer. *J Mammary Gland Biol Neoplasia* 1999; 4: 153–164.

[43] Srinivas G, Kusumakumary P, Nair MK, Panicker KR, Pillai MR. Bcl-2 protein and apoptosis in pediatric acute lymphoblastic leukaemia. *Int J Mol Med* 1998; 1: 755–759.

[44] Greenlund LJ, Korsmeyer SJ, Johnson EM Jr. Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. *Neuron* 1995; 15: 649–661.

[45] Memon SA, Moreno MB, Petrak D, Zacharchuk CM. Bcl-2 blocks glucocorticoid- but not Fas- or activation induced apoptosis in a T cell hybridoma. *J Immunol* 1995; 155: 4644–4652.

[46] Kobzdej M, Matuszyk J, Ziolo E, Strzadala L. Changes in glucocorticoid induced apoptosis and in expression of Bcl-2 protein during long term culture of thymic lymphoma. *Arch Immunol Ther Exp* 2000; 48: 13–16.

[47] Von Freeden-Jeffry U, Solvason N, Howard M, Murray R. The earliest T lineage-committed cells depend on IL-7 for Bcl 2 expression and normal cell cycle progression. *Immunity* 1997; 7: 147–154.

[48] Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 1994; 9: 1799–1805.

[49] Watabe M, Kawazoe N, Masuda Y, Nakajo S, Nakaya K. Bcl-2 protein inhibits bufalin-induced apoptosis through inhibition of mitogen-activated protein kinase activation in human leukemia U937 cells. *Cancer Res* 1997; 57: 3097–3100.

[50] Harada K, Ishibashi S, Miyashita T, Osuga J, Yagyu H, Ohashi K, Yazaki Y, Yamada N. Bcl-2 protein inhibits oxysterol-induced apoptosis through suppressing CPP32 mediated pathway. *FEBS Lett* 1997; 411: 63–66.

[51] Haldar S, Jena N, Croce CM. Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* 1995; 92: 4507–4511.

[52] Wang HG, Miyashita T, Takayama S, Sato T, Torigoe T, Krajewski S, Tanaka S, Hovey L 3rd, Troppmair J, Rapp UR, et al. Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. *Oncogene* 1994; 9: 2751–2756.

[53] Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; 90: 405–413.

[54] Hu Y, Benedict MA, Wu D, Inohara N, Nunez G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1 dependent caspase-9 activation. *Proc Natl Acad Sci USA* 1998; 95: 4386–4391.

[55] Pan G, O'Rourke K, Dixit VM. Caspase 9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 1998; 273: 5841–5845.

[56] Cosulich SC, Savory PJ, Clarke PR. Bcl-2 regulates amplification of caspase activation by cytochrome c. *Curr Biol* 1999; 9: 147–150.

[57] Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC. Inhibition of Bax channel-forming activity by Bcl-2. *Science* 1997; 277: 370–372.

[58] Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC. Channel formation by antiapoptotic protein Bcl 2. *Proc Natl Acad Sci USA* 1997; 94: 5113–5118.

[59] Schlesinger PH, Gross A, Yin XM, Yamamoto K, Saito M, Waksman G, Korsmeyer SJ. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc Natl Acad Sci USA* 1997; 94: 11357–11362.

[60] Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002; 2: 647–656.

[61] Grillot DA, Gonzalez-Garcia M, Ekhterae D, Duan L, Inohara N, Ohta S, Seldin MF, Nunez G. Genomic organization, promoter region analysis, and chromosome localization of the mouse bcl-x gene. *J Immunol* 1997; 158: 4750–4757.

[62] Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 1999; 399: 483–487.

[63] Von Ahsen O, Renken C, Perkins G, Kluck RM, Bossy-Wetzel E, Newmeyer DD. Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. *J Cell Biol* 2000; 150: 1027–1036.

[64] Gonzalez-Garcia M, Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, Nunez G. bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localises to mitochondria. *Development* 1994; 120: 3033–3042.

[65] Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993; 74: 597–608.

[66] Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am J Pathol* 1994; 145: 1323–1336.

[67] Yang XF, Weber GF, Cantor H. A novel Bcl-x isoform connected to the T cell receptor regulates apoptosis in T cells. *Immunity* 1997; 7: 629–639.

[68] Schorr K, Li M, Krajewski S, Reed JC, Furth PA. Bcl-2 gene family. *J Mammary Gland Biol Neoplasia* 1999; 4: 153–164.

[69] Hsu YT, Wolter KG, Youle RJ. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci USA* 1997; 94: 3668–3672.

[70] Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* 1998; 17: 3878–3885.

[71] Chou JJ, Li H, Salvesen GS, Yuan J, Wagner G. Solution structure of BID, an intracellular amplifier of apoptotic signalling. *Cell* 1999; 96: 615–624.

[72] Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M, Thompson CB. Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* 1997; 385: 353–357.

[73] Inohara N, Gourley TS, Carrio R, Muniz M, Merino J, Garcia I, Koseki T, Hu Y, Chen S, Nunez G. Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. *J Biol Chem* 1998; 273: 32479–32486.

[74] Song Q, Kuang Y, Dixit VM, Vincenz C. Boo, a novel negative regulator of cell death, interacts with Apaf-1. *EMBO J* 1999; 18: 167–178.

[75] Van Houten N, Blake SF, Li EJ, Hallam TA, Chilton DG, Gourley WK, Boise LH, Thompson CB, Thompson EB. Elevated expression of Bcl-2 and Bcl-x by intestinal intraepithelial lymphocytes: resistance to apoptosis by glucocorticoids and irradiation. *Int Immunol* 1997; 9: 945–953.

[76] Leverrier Y, Thomas J, Mathieu AL, Low W, Blanquier B, Marvel J. Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells. *Cell Death Differ* 1999; 6: 290–296.

[77] Rong P, Bennie AM, Epa WR, Barrett GL. Nerve growth factor determines survival and death of PC12 cells by regulation of the bcl-x, bax, and caspase-3 genes. *J Neurochem* 1999; 72: 2294–2300.

[78] Wang Z, Karras JG, Howard RG, Rothstein TL. Induction of bcl-x by CD40 engagement rescues sIg-induced apoptosis in murine B cells. *J Immunol* 1995; 155: 3722–3725.

[79] Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 1995; 3: 87–98.

[80] Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci USA* 1993; 90: 3516–3520.

[81] Bae J, Leo CP, Hsu SY, Hsueh AJ. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J Biol Chem* 2000; 275: 25255–25261.

[82] Rogers S, Wells R, Rechsteiner M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 1986; 234: 364–368.

[83] Akgul C, Turner PC, White MR, Edwards SW. Functional analysis of the human MCL-1 gene. *Cell Mol Life Sci* 2000; 57: 684–691.

[84] Yang T, Buchan HL, Townsend KJ, Craig RW. MCL-1, a member of the BLC-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. *J Cell Physiol* 1996; 166: 523–536.

[85] Townsend KJ, Trusty JL, Traupman MA, Eastman A, Craig RW. Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated pathway triggered through microtubule disruption and protein kinase C. *Oncogene* 1998; 17: 1223–1234.

[86] Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH. Suppression of PMN apoptosis by hypoxia is dependent on Mcl-1 and MAPK activity. *Surgery* 2000; 128: 171–177

[87] Boucher MJ, Morisset J, Vachon PH, Reed JC, Laine J, Rivard N. MEK/ERK signalling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. *J Cell Biochem* 2000; 79: 355–369.

[88] Leu CM, Chang C, Hu C. Epidermal growth factor (EGF) suppresses staurosporine-induced apoptosis by inducing mcl 1 via the mitogen activated protein kinase pathway. *Oncogene* 2000; 19: 1665–1675.

[89] Wang JM, Chao JR, Chen W, Kuo ML, Yen JJ, Yang-Yen HF. The antiapoptotic gene mcl 1 is up regulated by the phosphatidylinositol 3 kinase/Akt signalling pathway through a transcription factor complex containing CREB. *Mol Cell Biol* 1999; 19: 6195–6206

[90] Puthier D, Bataille R, Amiot M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK/STAT rather than ras/MAP kinase pathway. *Eur J Immunol* 1999; 29: 3945–3950.

[91] Epling Burnette PK, Liu JH, Catlett-Falcone R, Turkson J, Oshiro M, Kothapalli R, Li Y, Wang JM, Yang-Yen HF, Karras J, Jove R, Loughran TP Jr. Inhibition of STAT3 signalling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. *J Clin Invest* 2001; 107: 351–362.

[92] Epling Burnette PK, Zhong B, Bai F, Jiang K, Bailey RD, Garcia R, Jove R, Djeu JY, Loughran TP Jr, Wei S. Cooperative regulation of Mcl 1 by Janus kinase/stat and phosphatidylinositol 3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. *J Immunol* 2001; 166: 7486–7495.

[93] Liu H, Ma Y, Cole SM, Zander C, Chen KH, Karras J, Pope RM. Serine phosphorylation of STAT3 is essential for Mcl 1 expression and macrophage survival. *Blood* 2003; 102: 344–352.

[94] Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P, Whyte MK. Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death. *J Biol Chem* 2000; 275: 22136–22146.

[95] Haldar S, Jena N, Croce CM. Antiapoptosis potential of bcl-2 oncogene by dephosphorylation. *Biochem Cell Biol* 1994; 72: 455–462.

[96] Haldar S, Chintapalli J, Croce CM. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res* 1996; 56: 1253–1255.

[97] Ito T, Deng X, Carr B, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* 1997; 272: 11671–11673.

[98] Haldar S, Basu A, Croce CM. Bcl2 is the guardian of microtubule integrity. *Cancer Res* 1997; 57: 229–233

[99] Haldar S, Basu A, Croce CM. Serine 70 is one of the critical sites for drug-induced Bcl2 phosphorylation in cancer cells. *Cancer Res* 1998; 58: 1609–1615

[100] Deng X, Ito T, Carr B, Mumby M, May WS Jr. Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. *J Biol Chem* 1998; 273: 34157–34163

[101] Domina AM, Smith JH, Craig RW. Myeloid cell leukemia 1 is phosphorylated through two distinct pathways, one associated with extracellular signal-regulated kinase activation and the other with G2/M accumulation or protein phosphatase 1/2A inhibition. *J Biol Chem* 2000; 275: 21688–21694

[102] Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K, Reed JC. Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl 2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. *Am J Pathol* 1995; 146: 1309–1319.

[103] Craig RW. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. *Leukemia* 2002; 16: 444–454.

[104] Yang T, Kozopas KM, Craig RW. The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol* 1995; 128: 1173–1184.

[105] Zhou P, Qian L, Bieszczad CK, Noelle R, Binder M, Levy NB, Craig RW. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. *Blood* 1998; 92: 3226–3239.

[106] Sordet O, Bettaieb A, Bruey JM, Eymin B, Droin N, Ivarsson M, Garrido C, Solary E. Selective inhibition of apoptosis by TPA-induced differentiation of U937 leukemic cells. *Cell Death Differ* 1999; 6: 351–361.

[107] Lomo J, Smeland EB, Krajewski S, Reed JC, Blomhoff HK. Expression of the Bcl-2 homologue Mcl-1 correlates with survival of peripheral blood B lymphocytes. *Cancer Res* 1996; 56: 40–43.

[108] Lomo J, Blomhoff HK, Jacobsen SE, Krajewski S, Reed JC, Smeland EB. Interleukin-13 in combination with CD40 ligand potently inhibits apoptosis in human B lymphocytes: upregulation of Bcl-xL and Mcl-1. *Blood* 1997; 89: 4415–4424.

[109] Puthier D, Derenne S, Barille S, Moreau P, Harousseau JL, Bataille R, Amiot M. Mcl-1 and Bcl-xL are co-regulated by IL-6 in human myeloma cells. *Br J Haematol* 1999; 107: 392–395.

[110] Puthier D, Thabard W, Rapp M, Etrillard M, Harousseau J, Bataille R, Amiot M. Interferon alpha extends the survival of human myeloma cells through an upregulation of the Mcl-1 anti-apoptotic molecule. *Br J Haematol* 2001; 112: 358–363.

[111] Chao JR, Wang JM, Lee SF, Peng HW, Lin YH, Chou CH, Li JC, Huang HM, Chou CK, Kuo ML, Yen JJ, Yang-Yen HF. mcl-1 is an immediate-early gene activated by the granulocyte macrophage colony-stimulating factor (GM-CSF) signalling pathway and is one component of the GM-CSF viability response. *Mol Cell Biol* 1998; 18: 4883–4898.

[112] Moulding DA, Quayle JA, Hart CA, Edwards SW. Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood* 1998; 92: 2495–2502.

[113] Gibson L, Holmgreen SP, Huang DC, Bernard O, Copeland NG, Jenkins NA, Sutherland GR, Baker E, Adams JM, Cory S. bcl-w, a novel member of the bcl-2 family, promotes cell survival. *Oncogene* 1996; 13: 665–675.

[114] Holmgreen SP, Huang DC, Adams JM, Cory S. Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members. *Cell Death Differ* 1999; 6: 525–532.

[115] Hamner S, Skoglosa Y, Lindholm D. Differential expression of bcl-w and bcl-x messenger RNA in the developing and adult rat nervous system. *Neuroscience* 1999; 91: 673–684.

[116] Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N, de Kretser D, Metcalf D, Kontgen F, Adams JM, Cory S. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc Natl Acad Sci USA* 1998; 95: 12424–12431.

[117] Ross AJ, Waymire KG, Moss JE, Parlow AF, Skinner MK, Russell LD, MacGregor GR. Testicular degeneration in Bclw-deficient mice. *Nat Genet* 1998; 18: 251–256.

[118] O'Reilly LA, Print C, Hausmann G, Moriishi K, Cory S, Huang DC, Strasser A. Tissue expression and subcellular localization of the pro-survival molecule Bcl-w. *Cell Death Differ* 2001; 8: 486–494.

[119] Hinds MG, Lackmann M, Skea GL, Harrison PJ, Huang DC, Day CL. The structure of Bcl-w reveals a role for the C-terminal residues in modulating biological activity. *EMBO J* 2003; 22: 1497–1507.

[120] Wilson-Annan J, O'Reilly LA, Crawford SA, Hausmann G, Beaumont JG, Parma LP, Chen L, Lackmann M, Lithgow T, Hinds MG, Day CL, Adams JM, Huang DC. Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. *J Cell Biol* 2003; 162: 877–887.

[121] Kaufmann T, Schinzel A, Borner C. Bcl-w(edding) with mitochondria. *Trends Cell Biol* 2004; 14: 8–12.

[122] Yan W, Samson M, Jegou B, Toppari J. Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. *Mol Endocrinol* 2000; 14: 682–699.

[123] Kitamura S, Kondo S, Shinomura Y, Kanayama S, Miyazaki Y, Kiyohara T, Hiraoka S, Matsuzawa Y. Met/HGF receptor modulates bcl-w expression and inhibits apoptosis in human colorectal cancers. *Br J Cancer* 2000; 83: 668–673.

[124] Yang L, Zhang B, Toku K, Maeda N, Sakanaka M, Tanaka J. Improvement of the viability of cultured rat neurons by the non-essential amino acids L-serine and glycine that upregulates expression of the anti-apoptotic gene product Bcl-w. *Neurosci Lett* 2000; 295: 97–100.

[125] Yan C, Chen J, Chen D, Minami M, Pei W, Yin XM, Simon RP. Overexpression of the cell death suppressor Bcl-w in ischemic brain: implications for a neuroprotective role via the mitochondrial pathway. *J Cereb Blood Flow Metab* 2000; 20: 620–630.

[126] Lee R, Chen J, Matthews CP, McDougall JK, Neiman PE. Characterization of NR13-related human cell death regulator, Boo/Diva, in normal and cancer tissues. *Biochim Biophys Acta* 2001; 1520: 187–194.

[127] Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. *J Biol Chem* 1996; 271: 27201–27204.

[128] Kenny JJ, Knobloch TJ, Augustus M, Carter KC, Rosen CA, Lang JC. GRS, a novel member of the Bcl-2 gene family, is highly expressed in multiple cancer cell lines and in normal leukocytes. *Oncogene* 1997; 14: 997–1001.

[129] Karsan A, Yee E, Kaushansky K, Harlan JM. Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. *Blood* 1996; 87: 3089–3096.

[130] D'Sa-Eipper C, Chinnadurai G. Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities. *Oncogene* 1998; 16: 3105–3114.

[131] Choi SS, Park IC, Yun JW, Sung YC, Hong SI, Shin HS. A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. *Oncogene* 1995; 11: 1693–1698.

[132] Hatakeyama S, Hamasaki A, Negishi I, Loh DY, Sendo F, Nakayama K, Nakayama K. Multiple gene duplication and expression of mouse bcl-2-related genes, A1. *Int Immunol* 1998; 10: 631–637.

[133] Ko JK, Lee MJ, Cho SH, Cho JA, Lee BY, Koh JS, Lee SS, Shim YH, Kim CW. Bfl-1S, a novel alternative splice variant of Bfl-1, localises in the nucleus via its C-terminus and prevents cell death. *Oncogene* 2003; 22: 2457–2465.

[134] Wang XP, Hershman JM, Karsan A. TNF-alpha induction of A1 expression in human cancer cells. *Oncol Res* 1997, 9, 623–627.

[135] Chuang PI, Yee E, Karsan A, Winn RK, Harlan JM. A1 is a constitutive and inducible Bcl-2 homologue in mature human neutrophils. *Biochem Biophys Res Commun* 1998; 249: 361–365.

[136] Kremer L, Estaquier J, Brandt E, Ameisen JC, Locht C. Mycobacterium bovis Bacillus Calmette Guerin infection prevents apoptosis of resting human monocytes. *Eur J Immunol* 1997, 27: 2450–2456.

[137] Orlofsky A, Somogyi RD, Weiss LM, Prystowsky MB. The murine antiapoptotic protein A1 is induced in inflammatory macrophages and constitutively expressed in neutrophils. *J Immunol* 1999; 163: 412–419.

[138] Gross A, Terraza A, Ouahrani-Bettache S, Liautard JP, Dornand J. In vitro Brucella suis infection prevents the programmed cell death of human monocytic cells. *Infect Immun* 2000; 68: 342–351.

[139] Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 1998; 273: 13313–13316.

[140] Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS. STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. *Blood* 1997; 90: 1768–1776.

[141] Lee HH, Dadgostar H, Cheng Q, Shu J, Cheng G. NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signalling in B lymphocytes. *Proc Natl Acad Sci USA* 1999; 96: 9136–9141.

[142] Wang CY, Guttridge DC, Mayo MW, Baldwin AS Jr. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 1999; 19: 5923–5929.

[143] Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. *Genes Dev* 1999; 13: 382–387.

[144] Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev* 1999; 13: 400–411.

[145] Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, Korsmeyer SJ. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sci USA* 1995; 92: 7834–7838.

[146] Zhang H, Cowan-Jacob SW, Simonen M, Greenhalf W, Heim J, Meyhack B. Structural basis of BFL-1 for its interaction with BAX and its anti-apoptotic action in mammalian and yeast cells. *J Biol Chem* 2000; 275: 11092–11099.

[147] Duriez PJ, Wong F, Dorovini-Zis K, Shahidi R, Karsan A. A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. *J Biol Chem* 2000; 275: 18099–18107.

[148] Werner AB, de Vries E, Tait SW, Bontjer I, Borst J. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. *J Biol Chem* 2002; 277: 22781–22788.

[149] Apte SS, Mattei MG, Olsen BR. Mapping of the human BAX gene to chromosome 19q13.3-q13.4 and isolation of a novel alternatively spliced transcript, BAX delta. *Genomics* 1995; 26: 592–594.

[150] Cuddeback SM, Yamaguchi H, Komatsu K, Miyashita T, Yamada M, Wu C, Singh S, Wang HG. Molecular cloning and characterization of Bif-1. A novel Src homology 3 domain-containing protein that associates with Bax. *J Biol Chem* 2001; 276: 20559–20565.

[151] Zha H, Aime-Sempe C, Sato T, Reed JC. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. *J Biol Chem* 1996; 271: 7440–7444.

[152] Schmitt E, Paquet C, Beauchemin M, Dever-Bertrand J, Bertrand R. Characterization of Bax-sigma, a cell death-inducing isoform of Bax. *Biochem Biophys Res Commun* 2000; 270: 868–879.

[153] Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995; 80: 293–299.

[154] Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993; 74: 609–619.

[155] Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997; 139: 1281–1292.

[156] Nechushtan A, Smith CL, Hsu YT, Youle RJ. Conformation of the Bax C-terminus regulates subcellular location and cell death. *EMBO J* 1999; 18: 2330–2341.

[157] Suzuki M, Youle RJ, Tjandra N. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 2000; 103: 645–654.

[158] Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999; 13: 1899–1911.

[159] Antonsson B, Montessuit S, Sanchez B, Martinou JC. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* 2001; 276: 11615–11623.

[160] Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2002; 2: 183–192.

[161] Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002; 111: 331–342.

[162] Rostovtseva TK, Antonsson B, Suzuki M, Youle RJ, Colombini M, Bezrukov SM. *J Biol Chem* 2004; 279: 13575–13583.

[163] Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, Prevost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 1998; 281: 2027–2031.

[164] Martinou JC, Green DR. Breaking the mitochondrial barrier. *Nat Rev Mol Cell Biol* 2001; 2: 63–67.

[165] Roucou X, Montessuit S, Antonsson B, Martinou JC. Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein. *Biochem J* 2002; 368: 915–921.

[166] Zamzami N, Kroemer G. Apoptosis: mitochondrial membrane permeabilization— the (w)hole story? *Curr Biol* 2003; 13: R71–R73.

[167] Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* 1998; 17: 3878–3885.

[168] Hsu Y-T, Youle RJ. Nonionic detergents induce dimerization among members of the Bcl-2 family. *J Biol Chem* 1997; 272: 13829–13834.

[169] Xiang J, Chao DT, Korsmeyer SJ. BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc Natl Acad Sci USA* 1996; 93: 14559–14563.

[170] Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91: 479–489.

[171] Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R, Mazzei G, Nichols A, Martinou JC. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. *J Cell Biol* 1998, 143: 217–224.

[172] Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* 1999; 274: 2225–2233.

[173] Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, Prevost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 1998; 281: 2027–2031.

[174] Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998; 17: 37–49.

[175] Goping IS, Gross A, Lavoie JN, Nguyen M, Jemmerson R, Roth K, Korsmeyer SJ, Shore GC. Regulated targeting of BAX to mitochondria. *J Cell Biol* 1998; 143: 207–215.

[176] McCarthy NJ, Whyte MK, Gilbert CS, Evan GI. Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. *J Cell Biol* 1997, 136: 215–227.

[177] Lewis S, Bethell SS, Patel S, Martinou JC, Antonsson B. Purification and biochemical properties of soluble recombinant human Bax. *Protein Expr Purif* 1998; 13: 120–126.

[178] Dibbert B, Weber M, Nikolaizik WH, Vogt P, Schoni MH, Blaser K, Simon HU. Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation. *Proc Natl Acad Sci USA* 1999, 96: 13330–13335.

[179] Shinoura N, Yoshida Y, Asai A, Kirino T, Hamada H. Relative level of expression of Bax and Bcl-XL determines the cellular fate of apoptosis/necrosis induced by the overexpression of Bax. *Oncogene* 1999; 18: 5703–5713.

[180] Meijerink JP, Mensink EJ, Wang K, Sedlak TW, Sloetjes AW, de Witte T, Waksman G, Korsmeyer SJ. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. *Blood* 1998; 91: 2991–2997.

[181] Ulrich E, Kauffmann-Zeh A, Hueber AO, Williamson J, Chittenden T, Ma A, Evan G. Gene structure, cDNA sequence, and expression of murine Bak, a proapoptotic Bcl-2 family member. *Genomics* 1997; 44: 195–200.

[182] Herberg JA, Phillips S, Beck S, Jones T, Sheer D, Wu JJ, Prochazka V, Barr PJ, Kiefer MC, Trowsdale J. Genomic structure and domain organisation of the human Bak gene. *Gene* 1998; 211: 87–94.

[183] Kiefer MC, Brauer MJ, Powers VC, Wu JJ, Umansky SR, Tomei LD, Barr PJ. Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature* 1995; 374: 736–739.

[184] Pearson AS, Spitz FR, Swisher SG, Kataoka M, Sarkiss MG, Meyn RE, McDonnell TJ, Cristiano RJ, Roth JA. Up-regulation of the proapoptotic mediators Bax and Bak after adenovirus-mediated p53 gene transfer in lung cancer cells. *Clin Cancer Res* 2000; 6: 887–890.

[185] Leu JI, Dumont P, Hafey M, Murphy ME, George DL. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol* 2004; 6: 443–450.

[186] Pammer J, Weninger W, Ban J, Wojta J, Tschachler E. The cell death regulatory protein bak is expressed in endothelial cells in inflamed tissues and is induced by IFN-gamma in vitro. *Biochem Biophys Res Commun* 1999; 264: 139–143.

[187] Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 1997; 275: 983–986.

[188] Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* 2000; 14: 2060–2071.

[189] Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ* 2000; 7: 1166–1173.

[190] Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; 292: 727–730.
[191] Sun YF, Yu LY, Saarma M, Timmusk T, Arumae U. Neuron-specific Bcl-2 homology 3 domain-only splice variant of Bak is anti-apoptotic in neurons, but pro-apoptotic in non-neuronal cells. *J Biol Chem* 2001; 276: 16240–16247.
[192] Hsu SY, Kaipia A, McGee E, Lomeli M, Hsueh AJ. Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc Natl Acad Sci USA* 1997; 94: 12401–12406.
[193] Inohara N, Ekhterae D, Garcia I, Carrio R, Merino J, Merry A, Chen S, Nunez G. Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-XL. *J Biol Chem* 1998; 273: 8705–8710.
[194] Yang T, Kozopas KM, Craig RW. The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol* 1995; 128: 1173–1184.
[195] Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; 288: 1053–1058.
[196] Zhang H, Huang Q, Ke N, Matsuyama S, Hammock B, Godzik A, Reed JC. Drosophila pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *J Biol Chem* 2000; 275: 27303–27306.
[197] Hsu SY, Hsueh AJ. A splicing variant of the Bcl-2 member Bok with a truncated BH3 domain induces apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins in vitro. *J Biol Chem* 1998; 273: 30139–30146.
[198] Cai Z, Capoulade C, Moyret-Lalle C, Amor-Gueret M, Feunteun J, Larsen AK, Paillerets BB, Chouaib S. Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with loss of p53 function. *Oncogene* 1997; 15: 2817–2826.
[199] Yakovlev AG, Di Giovanni S, Wang G, Liu W, Stoica B, Faden AI. BOK and NOXA are essential mediators of p53-dependent apoptosis. *J Biol Chem* 2004; 279: 28367–28374.
[200] Brown GY, Bowers SJ, Loring G, Heberden C, Lee RM, Neiman PE. Role of Mtd/Bok in normal and neoplastic B cell development in the bursa of Fabricius. *Dev Comp Immunol* 2004; 28: 619–634.
[201] Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995; 80: 285–291.
[202] Kaipia A, Hsu SY, Hsueh AJ. Expression and function of a proapoptotic Bcl-2 family member Bcl-XL/Bcl-2-associated death promoter (BAD) in rat ovary. *Endocrinology* 1997; 138: 5497–5504.
[203] Tan Y, Demeter MR, Ruan H, Comb MJ. BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. *J Biol Chem* 2000; 275: 25865–25869.
[204] Zhou XM, Liu Y, Payne G, Lutz RJ, Chittenden T. Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. *J Biol Chem* 2000; 275: 25046–25051.
[205] Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell* 2000; 6: 41–51.
[206] Lizcano JM, Morrice N, Cohen P. Regulation of BAD by cAMP dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. *Biochem J* 2000; 349: 547–557.
[207] Virdee K, Parone PA, Tolkovsky AM. Phosphorylation of the pro-apoptotic protein BAD on serine 155, a novel site, contributes to cell survival. *Curr Biol* 2000; 10: 1151–1154.
[208] Dramsi S, Scheid MP, Maiti A, Hojabrpour P, Chen X, Schubert K, Goodlett DR, Aebersold R, Duronio V. Identification of a novel phosphorylation site, Ser-170, as a regulator of bad pro-apoptotic activity. *J Biol Chem* 2002; 277: 6399–6405.
[209] Zhang B, Zhang Y, Shacter E. Rac1 inhibits apoptosis in human lymphoma cells by stimulating Bad phosphorylation on Ser-75. *Mol Cell Biol* 2004; 24: 6205–6214.
[210] Won J, Kim DY, La M, Kim D, Meadows GG, Joe CO. Cleavage of 14-3-3 protein by caspase-3 facilitates bad interaction with Bcl-x(L) during apoptosis. *J Biol Chem* 2003; 278: 19347–19351.
[211] Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; 91: 231–241.
[212] Del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997; 278: 687–689.

[213] Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ, Greenberg ME. Survival factor mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell* 2002; 3: 631–643.

[214] Eisenmann KM, VanBrocklin MW, Staffend NA, Kitchen SM, Koo HM. Mitogen-activated protein kinase pathway dependent tumor-specific survival signalling in melanoma cells through inactivation of the proapoptotic protein Bad. *Cancer Res* 2003, 63: 8330–8337.

[215] Li WQ, Jiang Q, Khaled AR, Keller JR, Durum SK. Interleukin-7 inactivates the pro-apoptotic protein Bad promoting T cell survival. *J Biol Chem* 2004; 279: 29160–29166.

[216] Fang X, Yu S, Eder A, Mao M, Bast RC Jr, Boyd D, Mills GB. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncogene* 1999; 18: 6635–6640.

[217] Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, Klumpp S, Krieglstein J. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. *J Neurosci* 2002; 22: 3898–3909.

[218] Valks DM, Cook SA, Pham FH, Morrison PR, Clerk A, Sugden PH. Phenylephrine promotes phosphorylation of Bad in cardiac myocytes through the extracellular signal-regulated kinases 1/2 and protein kinase A. *J Mol Cell Cardiol* 2002, 34: 749–763.

[219] Cotteret S, Jaffer ZM, Beeser A, Chernoff J. p21 Activated kinase 5 (Pak5) localises to mitochondria and inhibits apoptosis by phosphorylating BAD. *Mol Cell Biol* 2003; 23: 5526–5539.

[220] Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999, 13: 2905–2927.

[221] Schurmann A, Mooney AF, Sanders LC, Sells MA, Wang HG, Reed JC, Bokoch GM. p21-activated kinase 1 phosphorylates the death agonist Bad and protects cells from apoptosis. *Mol Cell Biol* 2000; 20: 453–461.

[222] Jakobi R, Moertl E, Koeppel MA. p21-activated protein kinase gamma-PAK suppresses programmed cell death of BALB3T3 fibroblasts. *J Biol Chem* 2001; 276: 16624–16634.

[223] Gnesutta N, Qu J, Minden A. The serine/threonine kinase PAK4 prevents caspase activation and protects cells from apoptosis. *J Biol Chem* 2001; 276: 14414–14419.

[224] Cotteret S, Jaffer ZM, Beeser A, Chernoff J. p21-activated kinase 5 (Pak5) localises to mitochondria and inhibits apoptosis by phosphorylating BAD. *Mol Cell Biol* 2003; 23: 5526–5539.

[225] Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; 91: 231–241.

[226] Blume-Jensen P, Janknecht R, Hunter T. The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. *Curr Biol* 1998; 8: 779–782.

[227] Harada H, Becknell B, Wilm M, Mann M, Huang LJ, Taylor SS, Scott JD, Korsmeyer SJ. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. *Mol Cell* 1999; 3: 413–422.

[228] Yan B, Zemskova M, Holder S, Chin V, Kraft A, Koskinen PJ, Lilly M. The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death. *J Biol Chem* 2003, 278: 45358–45367.

[229] Taghiyev AF, Guseva NV, Harada H, Knudson CM, Rokhlin OW, Cohen MB. Overexpression of BAD potentiates sensitivity to tumor necrosis factor-related apoptosis-inducing ligand treatment in the prostatic carcinoma cell line LNCaP. *Mol Cancer Res* 2003; 1: 500–507.

[230] Seo SY, Chen YB, Ivanovska I, Ranger AM, Hong SJ, Dawson VL, Korsmeyer SJ, Bellows DS, Fannjiang Y, Hardwick JM. BAD is a pro-survival factor prior to activation of its pro-apoptotic function. *J Biol Chem* 2004; 279: 42240–42249.

[231] Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ. BID: a novel BH3 domain-only death agonist. *Genes Dev* 1996, 10: 2859–2869.

[232] Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; 94: 491–501.

[233] Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999; 144: 891–901.

[234] Schendel SL, Azimov R, Pawlowski K, Godzik A, Kagan BL, Reed JC. Ion channel activity of the BH3 only Bcl-2 family member, BID. *J Biol Chem* 1999; 274: 21932–21936.

[235] Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; 94: 481–490.

[236] Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 1996; 86: 147–157.

[237] Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 1998; 60: 619–642.

[238] Renshaw SA, Dempsey CE, Barnes FA, Bagstaff SM, Dower SK, Bingle CD, Whyte MK. Three novel Bid proteins generated by alternative splicing of the human Bid gene. *J Biol Chem* 2004; 279: 2846–2855.

[239] Muravenko OV, Kashuba VI, Kutsenko AS, Gizatullin RZ, Protopopov AI, Kvasha SM, Al-Amin AN, Zelenin AV, Zabarovsky ER. Human HRK gene maps to position 12q13.1. *Chromosome Res* 2000; 8: 656.

[240] Inohara N, Ding L, Chen S, Nunez G. harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival promoting proteins Bcl-2 and Bcl-X(L). *EMBO J* 1997; 16: 1686–1694.

[241] Imaizumi K, Tsuda M, Imai Y, Wanaka A, Takagi T, Tohyama M. Molecular cloning of a novel polypeptide, DP5, induced during programmed neuronal death. *J Biol Chem* 1997; 272: 18842–18848.

[242] Sanz C, Benito A, Inohara N, Ekhterae D, Nunez G, Fernandez-Luna JL. Specific and rapid induction of the proapoptotic protein Hrk after growth factor withdrawal in hematopoietic progenitor cells. *Blood* 2000; 95: 2742–2747.

[243] Imaizumi K, Morihara T, Mori Y, Katayama T, Tsuda M, Furuyama T, Wanaka A, Takeda M, Tohyama M. The cell death-promoting gene DP5, which interacts with the BCL2 family, is induced during neuronal apoptosis following exposure to amyloid beta protein. *J Biol Chem* 1999; 274: 7975–7981.

[244] Harris CA, Johnson EM Jr. BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. *J Biol Chem* 2001; 276: 37754–37760.

[245] Shinoe T, Wanaka A, Nikaido T, Kanazawa K, Shimizu J, Imaizumi K, Kanazawa I. Upregulation of the pro-apoptotic BH3-only peptide harakiri in spinal neurons of amyotrophic lateral sclerosis patients. *Neurosci Lett* 2001; 313: 153–157.

[246] Wakabayashi T, Kosaka J, Hommura S. Up-regulation of Hrk, a regulator of cell death, in retinal ganglion cells of axotomized rat retina. *Neurosci Lett* 2002; 318: 77–80.

[247] Sanz C, Benito A, Inohara N, Ekhterae D, Nunez G, Fernandez-Luna JL. Specific and rapid induction of the proapoptotic protein Hrk after growth factor withdrawal in hematopoietic progenitor cells. *Blood* 2000; 95: 2742–2747.

[248] Jurisicova A, Antenos M, Varmuza S, Tilly JL, Casper RF. Expression of apoptosis-related genes during human preimplantation embryo development: potential roles for the Harakiri gene product and Caspase-3 in blastomere fragmentation. *Mol Hum Reprod* 2003; 9: 133–141.

[249] Sanz C, Mellstrom B, Link WA, Naranjo JR, Fernandez-Luna JL. Interleukin 3-dependent activation of DREAM is involved in transcriptional silencing of the apoptotic Hrk gene in hematopoietic progenitor cells. *EMBO J* 2001; 20: 2286–2292.

[250] Sunayama J, Ando Y, Itoh N, Tomiyama A, Sakurada K, Sugiyama A, Kang D, Tashiro F, Gotoh Y, Kuchino Y, Kitanaka C. Physical and functional interaction between BH3-only protein Hrk and mitochondrial pore-forming protein p32. *Cell Death Differ* 2004; 11: 771–781.

[251] Bouillet P, Zhang LC, Huang DC, Webb GC, Bottema CD, Shore P, Eyre HJ, Sutherland GR, Adams JM. Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim. *Mamm Genome* 2001; 12: 163–168.

[252] Hsu SY, Lin P, Hsueh AJ. BOD (Bcl-2-related ovarian death gene) is an ovarian BH3 domain-containing proapoptotic Bcl-2 protein capable of dimerization with diverse antiapoptotic Bcl-2 members. *Mol Endocrinol* 1998; 12: 1432–1440.

[253] O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 1998; 17: 384–395.

[254] O'Reilly LA, Cullen L, Visvader J, Lindeman GJ, Print C, Bath ML, Huang DC, Strasser A. The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. *Am J Pathol* 2000; 157: 449–461.

[255] Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, Strasser A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; 286: 1735–1738.

[256] Bouillet P, Huang DC, O'Reilly LA, Puthalakath H, O'Connor L, Cory S, Adams JM, Strasser A. The role of the pro-apoptotic Bcl-2 family member bim in physiological cell death. *Ann NY Acad Sci* 2000; 926: 83–89.

[257] Shinjyo T, Kuribara R, Inukai T, Hosoi H, Kinoshita T, Miyajima A, Houghton PJ, Look AT, Ozawa K, Inaba T. Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signalling in murine hematopoietic progenitors. *Mol Cell Biol* 2001; 21: 854–864.

[258] Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* 2001; 29: 629–643.

[259] Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM. Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 2001; 29: 615–628.

[260] Marani M, Miyashita T, Shikama Y, Tadokoro K, Yamada M. Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl-2 family. *FEBS Lett* 2001; 509: 135–141.

[261] Liu JW, Chandra D, Tang SH, Chopra D, Tang DG. Identification and characterization of Bimgamma, a novel proapoptotic BH3-only splice variant of Bim. *Cancer Res* 2002; 62: 2976–2981.

[262] Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999; 3: 287–296.

[263] Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* 2000; 10: 1201–1204.

[264] Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM. Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 2001; 29: 615–628.

[265] Strasser A, Puthalakath H, Bouillet P, Huang DC, O'Connor L, O'Reilly LA, Cullen L, Cory S, Adams JM. The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. *Ann NY Acad Sci* 2000; 917: 541–548.

[266] Ley R, Ewings KE, Hadfield K, Howes E, Balmanno K, Cook SJ. Extracellular signal-regulated kinases 1/2 are serum-stimulated "Bim(EL) kinases" that bind to the BH3-only protein Bim(EL) causing its phosphorylation and turnover. *J Biol Chem* 2004; 279: 8837–8847.

[267] Chen D, Zhou Q. Caspase cleavage of BimEL triggers a positive feedback amplification of apoptotic signaling. *Proc Natl Acad Sci USA* 2004; 101: 1235–1240.

[268] Luciano F, Jacquel A, Colosetti P, Herrant M, Cagnol S, Pages G, Auberger P. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* 2003; 22: 6785–6793.

[269] Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM Jr. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* 2003; 38: 899–914.

[270] Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, Marrack P. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity* 2002; 16: 759–767.

[271] Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 2002; 9: 505–512.

[272] Hildeman DA, Zhu Y, Mitchell TC, Kappler J, Marrack P. Molecular mechanisms of activated T cell death in vivo. *Curr Opin Immunol* 2002; 14: 354–359.

[273] Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* 2001; 29: 629–643.

[274] Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM. Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 2001; 29: 615–628.

[275] Harris CA, Johnson EM Jr. BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. *J Biol Chem* 2001; 276: 37754–37760.

[276] Foncea R, Galvez A, Perez V, Morales MP, Calixto A, Melendez J, Gonzalez-Jara F, Diaz-Araya G, Sapag-Hagar M, Sugden PH, LeRoith D, Lavandero S. Extracellular regulated kinase, but not protein kinase C, is an antiapoptotic signal of insulin-like growth factor 1 on cultured cardiac myocytes. *Biochem Biophys Res Commun* 2000; 273: 736–744.

[277] Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* 2003; 5: 733–740.

[278] Weston CR, Balmanno K, Chalmers C, Hadfield K, Molton SA, Ley R, Wagner EF, Cook SJ. Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways. *Oncogene* 2003; 22: 1281–1293.

[279] Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffer PJ. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol* 2002; 156: 531–542.

[280] Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM, Medema RH. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol* 2002; 168: 5024–5031.

[281] Sandalova E, Wei CH, Masucci MG, Levitsky V. Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering. *Proc Natl Acad Sci USA* 2004; 101: 3011–3016.

[282] Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, D'Sa-Eipper C, Chinnadurai G. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell* 1994; 79: 341–351.

[283] Yasuda M, D'Sa-Eipper C, Gong XL, Chinnadurai G. Regulation of apoptosis by a Caenorhabditis elegans BNIP3 homolog. *Oncogene* 1998; 17: 2525–2530.

[284] Cizeau J, Ray R, Chen G, Gietz RD, Greenberg AH. The C. elegans orthologue ceBNIP3 interacts with CED-9 and CED-3 but kills through a BH3- and caspase-independent mechanism. *Oncogene* 2000; 19: 5453–5463.

[285] Zhang H, Heim J, Meyhack B. Novel BNIP1 variants and their interaction with BCL2 family members. *FEBS Lett* 1999; 448: 23–27.

[286] Yasuda M, Chinnadurai G. Functional identification of the apoptosis effector BH3 domain in cellular protein BNIP1. *Oncogene* 2000; 19: 2363–2367.

[287] Garnier M, Di Lorenzo D, Albertini A, Maggi A. Identification of estrogen-responsive genes in neuroblastoma SK-ER3 cells. *J Neurosci* 1997; 17: 4591–4599.

[288] Zhou YT, Soh UJ, Shang X, Guy GR, Low BC. The BNIP-2 and Cdc42GAP homology/Sec14p-like domain of BNIP-Salpha is a novel apoptosis-inducing sequence. *J Biol Chem* 2002; 277: 7483–7492.

[289] Zhang HM, Yanagawa B, Cheung P, Luo H, Yuan J, Chau D, Wang A, Bohunek L, Wilson JE, McManus BM, Yang D. Nip21 gene expression reduces coxsackievirus B3 replication by promoting apoptotic cell death via a mitochondria-dependent pathway. *Circ Res* 2002; 90: 1251–1258.

[290] Chen G, Cizeau J, Vande Velde C, Park JH, Bozek G, Bolton J, Shi L, Dubik D, Greenberg A. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. *J Biol Chem* 1999; 274: 7–10.

[291] Matsushima M, Fujiwara T, Takahashi E, Minaguchi T, Eguchi Y, Tsujimoto Y, Suzumori K, Nakamura Y. Isolation, mapping, and functional analysis of a novel human cDNA (BNIP3L) encoding a protein homologous to human NIP3. *Genes Chromosomes Cancer* 1998; 21: 230–235.

[292] Shen L, Hu J, Lu H, Wu M, Qin W, Wan D, Li YY, Gu J. The apoptosis-associated protein BNIPL interacts with the two cell proliferation-related proteins, MIF and GFER. *FEBS Lett* 2003; 540: 86–90.

[293] Qin W, Hu J, Guo M, Xu J, Li J, Yao G, Zhou X, Jiang H, Zhang P, Shen L, Wan D, Gu J. BNIPL-2, a novel homologue of BNIP-2, interacts with Bcl-2 and Cdc42GAP in apoptosis. *Biochem Biophys Res Commun* 2003; 308: 379–385.

[294] Farooq M, Kim Y, Im S, Chung E, Hwang S, Sohn M, Kim M, Kim J. Cloning of BNIP3h, a member of proapoptotic BNIP3 family genes. *Exp Mol Med* 2001; 33: 169–173.

[295] Yasuda M, Theodorakis P, Subramanian T, Chinnadurai G. Adenovirus E1B-19K/BCL-2 interacting protein BNIP3 contains a BH3 domain and a mitochondrial targeting sequence. *J Biol Chem* 1998; 273: 12415–12421.

[296] Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, Saxena S, Gietz RD, Greenberg AH. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. *J Exp Med* 1997; 186: 1975–1983.

[297] Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, Greenberg AH. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 2000; **20**: 5454–5468.

[298] Verma S, Budarf ML, Emanuel BS, Chinnadurai G. Structural analysis of the human pro-apoptotic gene Bik: chromosomal localization, genomic organization and localization of promoter sequences. *Gene* 2000; **254**: 157–162.

[299] Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, Ebb RG, Subramanian T, Chittenden T, Lutz RJ, et al. Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* 1995; **11**: 1921–1928.

[300] Daniel PT, Pun KT, Ritschel S, Sturm I, Holler J, Dorken B, Brown R. Expression of the death gene Bik/Nbk promotes sensitivity to drug-induced apoptosis in corticosteroid-resistant T-cell lymphoma and prevents tumor growth in severe combined immunodeficient mice. *Blood* 1999; **94**: 1100–1107.

[301] Mathai JP, Germain M, Marcellus RC, Shore GC. Induction and endoplasmic reticulum location of BIK/NBK in response to apoptotic signalling by E1A and p53. *Oncogene* 2002; **21**: 2534–2544.

[302] Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, D'Sa-Eipper C, Chinnadurai G. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell* 1994; **79**: 341–351.

[303] Han J, Sabbatini P, White E. Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. Mol Cell Biol 1996; **16**: 5857–5864.

[304] Elangovan B, Chinnadurai G. Functional dissection of the pro-apoptotic protein Bik. Heterodimerization with anti-apoptosis proteins is insufficient for induction of cell death. *J Biol Chem* 1997; **272**: 24494–24498.

[305] Verma S, Zhao LJ, Chinnadurai G. Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis. *J Biol Chem* 2001; **276**: 4671–4676.

[306] Orth K, Dixit VM. Bik and Bak induce apoptosis downstream of CrmA but upstream of inhibitor of apoptosis. *J Biol Chem* 1997; **272**: 8841–8844.

[307] Jiang A, Clark EA. Involvement of bik, a proapoptotic member of the bcl-2 family, in surface igm-mediated b cell apoptosis. *J Immunol* 2001; **166**: 6025–6033.

[308] Marshansky V, Wang X, Bertrand R, Luo H, Duguid W, Chinnadurai G, Kanaan N, Vu MD, Wu J. Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. *J Immunol* 2001; **166**: 3130–3142.

[309] Hegde R, Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Blk, a BH3-containing mouse protein that interacts with Bcl-2 and Bcl-xL, is a potent death agonist. *J Biol Chem* 1998; **273**: 7783–7786.

[310] Paquet C, Schmitt E, Beauchemin M, Bertrand R. Activation of multidomain and BH3-only pro-apoptotic Bcl-2 family members in p53 defective cells. *Apoptosis* 2004; **9**: 815–831.

[311] Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; **288**: 1053–1058.

[312] Eferl R, Ricci R, Kenner L, Zenz R, David JP, Rath M, Wagner EF. Liver tumor development. c-Jun antagonizes the proapoptotic activity of p53. *Cell* 2003; **112**: 181–192.

[313] Flores ER, Tsai KY, Crowley D, Sengupta S, Yang A, McKeon F, Jacks T. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* 2002; **416**: 560–564.

[314] Seo YW, Shin JN, Ko KH, Cha JH, Park JY, Lee BR, Yun CW, Kim YM, Seol DW, Kim DW, Yin XM, Kim TH. The molecular mechanism of Noxa-induced mitochondrial dysfunction in p53-mediated cell death. *J Biol Chem* 2003; **278**: 48292–48299.

[315] Kim JY, Ahn HJ, Ryu JH, Suk K, Park JH. BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1alpha. *J Exp Med* 2004; **199**: 113–124.

[316] Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DC, Strasser A. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001; **293**: 1829–1832.

[317] Day CL, Puthalakath H, Skea G, Strasser A, Barsukov I, Lian LY, Huang DC, Hinds MG. Localization of dynein light chains 1 and 2 and their pro-apoptotic ligands. *Biochem J* 2004; **377**: 597–605.

[318] Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999; 3: 287–296.

[319] Lei K, Davis RJ. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci USA* 2003; 100: 2432–2437.

[320] Morales AA, Olsson A, Celsing F, Osterborg A, Jondal M, Osorio LM. Expression and transcriptional regulation of functionally distinct Bmf isoforms in B chronic lymphocytic leukemia cells. *Leukemia* 2004; 18: 41–47.

[321] Miura I, Siegfried JM, Resau J, Keller SM, Zhou JY, Testa JR. Chromosome alterations in 21 non-small cell lung carcinomas. *Genes Chromosomes Cancer* 1990; 2: 328–338.

[322] Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. *Cancer Res* 1994; 54: 1152–1155.

[323] Johns MM 3rd, Westra WH, Califano JA, Eisele D, Koch WM, Sidransky D. Allelotype of salivary gland tumors. *Cancer Res* 1996; 56: 1151–1154.

[324] Mutirangura A, Tanunyutthawongese C, Pornthanakasem W, Kerekhanjanarong V, Sriuranpong V, Yenrudi S, Supiyaphun P, Voravud N. Genomic alterations in nasopharyngeal carcinoma: loss of heterozygosity and Epstein-Barr virus infection. *Br J Cancer* 1997; 76: 770–776.

[325] Han J, Flemington C, Houghton AB, Gu Z, Zambetti GP, Lutz RJ, Zhu L, Chittenden T. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. *Proc Natl Acad Sci USA* 2001; 98: 11318–11323.

[326] Sanchez-Cespedes M, Ahrendt SA, Piantadosi S, Rosell R, Monzo M, Wu L, Westra WH, Yang SC, Jen J, Sidransky D. Chromosomal alterations in lung adenocarcinoma from smokers and nonsmokers. *Cancer Res* 2001; 61: 1309–1313.

[327] Hoque MO, Begum S, Sommer M, Lee T, Trink B, Ratovitski E, Sidransky D. PUMA in head and neck cancer. *Cancer Lett* 2003; 199: 75–81.

[328] Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 2001; 7: 673–682.

[329] Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001; 7: 683–694.

[330] Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM, Strasser A. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003; 302: 1036–1038.

[331] Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL, Zambetti GP. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 2003; 4: 321–328.

[332] Liu FT, Newland AC, Jia L. Bax conformational change is a crucial step for PUMA-mediated apoptosis in human leukemia. *Biochem Biophys Res Commun* 2003; 310: 956–962.

[333] Nagase T, Seki N, Ishikawa K, Tanaka A, Nomura N. Prediction of the coding sequences of unidentified human genes. V. The coding sequences of 40 new genes (KIAA0161-KIAA0200) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res* 1996; 3: 17–24.

[334] Kasof GM, Goyal L, White E. Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. *Mol Cell Biol* 1999; 19: 4390–4404.

[335] Gharavi AG, Yan Y, Scolari F, Schena FP, Frasca GM, Ghiggeri GM, Cooper K, Amoroso A, Viola BF, Battini G, Caridi G, Canova C, Farhi A, Subramanian V, Nelson-Williams C, Woodford S, Julian BA, Wyatt RJ, Lifton RP. IgA nephropathy, the most common cause of glomerulonephritis, is linked to 6q22–23. *Nat Genet* 2000; 26: 354–357.

[336] Haraguchi T, Holaska JM, Yamane M, Koujin T, Hashiguchi N, Mori C, Wilson KL, Hiraoka Y. Emerin binding to Btf, a death-promoting transcriptional repressor, is disrupted by a missense mutation that causes Emery-Dreifuss muscular dystrophy. *Eur J Biochem* 2004; 271: 1035–1045.

[337] Kataoka T, Holler N, Micheau O, Martinon F, Tinel A, Hofmann K, Tschopp J. Bcl-rambo, a novel Bcl-2 homologue that induces apoptosis via its unique C-terminal extension. *J Biol Chem* 2001; 276: 19548–19554.

[338] Yi P, Zhang W, Zhai Z, Miao L, Wang Y, Wu M. Bcl-rambo beta, a special splicing variant with an insertion of an Alu-like cassette, promotes etoposide- and Taxol-induced cell death. *FEBS Lett* 2003; 534: 61–68.

[339] Guo B, Godzik A, Reed JC. Bcl-G, a novel pro-apoptotic member of the Bcl-2 family. *J Biol Chem* 2001; 276: 2780–2785.

[340] Montpetit A, Boily G, Sinnett D. A detailed transcriptional map of the chromosome 12p12 tumour suppressor locus. *Eur J Hum Genet* 2002; 10: 62–71.

[341] Nakamura M, Tanigawa Y. Characterization of ubiquitin-like polypeptide acceptor protein, a novel pro-apoptotic member of the Bcl2 family. *Eur J Biochem* 2003; 270: 4052–4058.

[342] Tan KO, Tan KM, Chan SL, Yee KS, Bevort M, Ang KC, Yu VC. MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. *J Biol Chem* 2001; 276: 2802–2807.

[343] Aouacheria A, Arnaud E, Venet S, Lalle P, Gouy M, Rigal D, Gillet G. Nrh, a human homologue of Nr-13 associates with Bcl-Xs and is an inhibitor of apoptosis. *Oncogene* 2001; 20: 5846–5855.

[344] Ke N, Godzik A, Reed JC. Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak. *J Biol Chem* 2001; 276: 12481–12484.

[345] Zhai D, Ke N, Zhang H, Ladror U, Joseph M, Eichinger A, Godzik A, Ng SC, Reed JC. Characterization of the anti-apoptotic mechanism of Bcl-B. *Biochem J* 2003; 376: 229–236.

[346] Zhang H, Holzgreve W, De Geyter C. Bcl2-L-10, a novel anti-apoptotic member of the Bcl-2 family, blocks apoptosis in the mitochondria death pathway but not in the death receptor pathway. *Hum Mol Genet* 2001; 10: 2329–2339.

[347] Rogers S, Wells R, Rechsteiner M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 1986; 234: 364.

[348] Shibasaki F, Kondo E, Akagi T, McKeon F. Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and BCL-2. *Nature* 1997; 386: 728–731.

[349] Wang HG, Reed JC. BCL-2, RAF-1 and mitochondrial regulation of apoptosis. *Biofactors* 1998; 8: 13–16.

[350] Huang DC, Adams JM, Cory S. The conserved N-terminal BH4 domain of BCL-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J* 1998; 117: 1029–1103.

[351] Scorilas A, Kyriakopoulou L, Yousef GM, Ashworth LK, Kwamie A, Diamandis EP. Molecular cloning, physical mapping, and expression analysis of a novel gene, BCL2L12, encoding a proline-rich protein with a highly conserved BH2 domain of the Bcl-2 family. *Genomics* 2001; 72: 217–221.

[352] Hirai H, Varmus HE. Site-directed mutagenesis of the SH2- and SH3-coding domains of c-src produces varied phenotypes, including oncogenic activation of p60c-src. *Mol Cell Biol* 1990; 10: 1307–1318.

[353] Jackson LC, Hotchkiss RN. Cubital tunnel surgery. Complications and treatment of failures. *Hand Clin* 1996; 12: 449–456.

[354] Coultas L, Pellegrini M, Visvader JE, Lindeman GJ, Chen L, Adams JM, Huang DC, Strasser A. Bfk: a novel weakly proapoptotic member of the Bcl-2 protein family with a BH3 and a BH2 region. *Cell Death Differ* 2003; 10: 185–92.

[355] Satou T, Cummings BJ, Cotman CW. Immunoreactivity for Bcl-2 protein within neurons in the Alzheimer's disease brain increases with disease severity. *Brain Res* 1995; 697: 35–43.

[356] Jarskog LF, Gilmore JH. Developmental expression of Bcl-2 protein in human cortex. *Brain Res Dev Brain Res* 2000; 119: 225–230.

[357] Tan J, Town T, Placzek A, Kundtz A, Yu H, Mullan M. Bcl-X(L) inhibits apoptosis and necrosis produced by Alzheimer's beta-amyloid1-40 peptide in PC12 cells. *Neurosci Lett* 1999; 272: 5–8.

[358] Engidawork E, Gulesserian T, Seidl R, Cairns N, Lubec G. Expression of apoptosis related proteins in brains of patients with Alzheimer's disease. *Neurosci Lett* 2001; 303: 79–82.

[359] Hirsch EC, Hunot S, Faucheux B, Agid Y, Mizuno Y, Mochizuki H, Tatton WG, Tatton N, Olanow WC. Dopaminergic neurons degenerate by apoptosis in Parkinson's disease. *Mov Disord* 1999; 14: 383–385.

[360] Mogi M, Harada M, Kondo T, Mizuno Y, Narabayashi H, Riederer P, Nagatsu T. bcl-2 protein is increased in the brain from parkinsonian patients. *Neurosci Lett* 1996; 215: 137–139.

[361] Weiss RA. How does HIV cause AIDS? *Science* 1993; 260: 1273–1279.

[362] Hashimoto F, Oyaizu N, Kalyanaraman VS, Pahwa S. Modulation of Bcl-2 protein by CD4 cross-linking: a possible mechanism for lymphocyte apoptosis in human immunodeficiency virus infection and for rescue of apoptosis by interleukin-2. *Blood* 1997; 90: 745–753.

[363] Regamey N, Harr T, Battegay M, Erb P. Downregulation of Bcl-2, but not of Bax or Bcl-x, is associated with T lymphocyte apoptosis in HIV infection and restored by antiretroviral therapy or by interleukin 2. *AIDS Res Hum Retroviruses* 1999; 15: 803–810.

[364] Re M, Gibellini D, Aschbacher R, Vignoli M, Furlini G, Ramazzotti E, Bertolaso L, La Placa M. High levels of HIV-1 replication show a clear correlation with downmodulation of Bcl-2 protein in peripheral blood lymphocytes of HIV-1-seropositive subjects. *J Med Virol* 1998; 56: 66–73.

[365] French LE, Hahne M, Viard I, Radlgruber G, Zanone R, Becker K, Muller C, Tschopp J. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *J Cell Biol* 1996; 133: 335–343.

[366] Komaki S, Kohno M, Matsuura N, Shimadzu M, Adachi N, Hoshide R, Nishiyama S, Matsuda I. The polymorphic 43Thr bcl-2 protein confers relative resistance to autoimmunity: an analytical evaluation. *Hum Genet* 1998; 103: 435–440.

[367] Guo K, Searfoss G, Krolikowski D, Pagnoni M, Franks C, Clark K, Yu KT, Jaye M, Ivashchenko Y. Hypoxia induces the expression of the pro-apoptotic gene BNIP3. *Cell Death Differ* 2001; 8: 367–376.

[368] Graham RM, Frazier DP, Thompson JW, Haliko S, Li H, Wasserlauf BJ, Shiga MG, Bishopric NH, Webster KA. A unique pathway of cardiac myocyte death caused by hypoxia-acidosis. *J Exp Biol* 2004; 207: 3189–3200.

[369] Arscott PL, Baker JR Jr. Apoptosis and thyroiditis. *Clin Immunol Immunopathol* 1998; 87: 207–217.

[370] Stassi G, Todaro M, Bucchieri F, Stoppacciaro A, Farina F, Zummo G, Testi R, De Maria R. Fas/Fas ligand driven T cell apoptosis as a consequence of ineffective thyroid immunoprivilege in Hashimoto's thyroiditis. *J Immunol* 1999; 162: 263–267.

[371] Muller-Hocker J. Expression of bcl-2, Bax and Fas in oxyphil cells of Hashimoto thyroiditis. *Virchows Arch* 2000; 436: 602–607.

[372] Lamhamedi-Cherradi SE, Luan JJ, Eloy L, Fluteau G, Bach JF, Garchon HJ. Resistance of T-cells to apoptosis in autoimmune diabetic (NOD) mice is increased early in life and is associated with dysregulated expression of Bcl-x. *Diabetologia* 1998; 41: 178–184.

[373] Adachi M, Tefferi A, Greipp PR, Kipps TJ, Tsujimoto Y. Preferential linkage of bcl-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. *J Exp Med* 1990; 171: 559–564.

[374] Ayhan A, Yasui W, Yokozaki H, Seto M, Ueda R, Tahara E. Loss of heterozygosity at the bcl-2 gene locus and expression of bcl-2 in human gastric and colorectal carcinomas. *Jpn J Cancer Res* 1994; 85: 584–591.

[375] Kondo E, Yoshino T, Yamadori I, Matsuo Y, Kawasaki N, Minowada J, Akagi T. Expression of Bcl-2 protein and Fas antigen in non-Hodgkin's lymphomas. *Am J Pathol* 1994; 145: 330–337.

[376] Binder C, Marx D, Overhoff R, Binder L, Schauer A, Hiddemann W. Bcl-2 protein expression in breast cancer in relation to established prognostic factors and other clinicopathological variables. *Ann Oncol* 1995; 6: 1005–1010.

[377] Ramsay JA, From L, Kahn HJ. bcl-2 protein expression in melanocytic neoplasms of the skin. *Mod Pathol* 1995; 8: 150–154.

[378] Coustan-Smith E, Kitanaka A, Pui CH, McNinch L, Evans WE, Raimondi SC, Behm FG, Arico M, Campana D. Clinical relevance of BCL-2 overexpression in childhood acute lymphoblastic leukemia. *Blood* 1996; 87: 1140–1146.

[379] Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, Kitada S, Reed JC. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. *Am J Pathol* 1996; 148: 1567–1576.

[380] Flohil CC, Janssen PA, Bosman FT. Expression of Bcl-2 protein in hyperplastic polyps, adenomas, and carcinomas of the colon. *J Pathol* 1996; 178: 393–397.

[381] Tsuji M, Murakami Y, Kanayama H, Sano T, Kagawa S. Immunohistochemical analysis of Ki-67 antigen and Bcl-2 protein expression in prostate cancer: effect of neoadjuvant hormonal therapy. *Br J Urol* 1998; 81: 116–121.

[382] Malkinson AM. Molecular comparison of human and mouse pulmonary adenocarcinomas. *Exp Lung Res* 1998; 24: 541–555.

[383] Zaja F, Di Loreto C, Amoroso V, Salmaso F, Russo D, Silvestri F, Fanin R, Damiani D, Infanti L, Mariuzzi L, Beltrami CA, Baccarani M. BCL-2 immunohistochemical evaluation in B cell chronic lymphocytic leukemia and hairy cell leukemia before treatment with fludarabine and 2-chloro-deoxy-adenosine. *Leuk Lymphoma* 1998; 28: 567–572.

[384] Laudanski J, Chyczewski L, Niklinska WE, Kretowska M, Furman M, Sawicki B, Niklinski J. Expression of bcl-2 protein in non small cell lung cancer: correlation with clinicopathology and patient survival. *Neoplasma* 1999; 46: 25–30.

[385] Gobe G, Rubin M, Williams G, Sawczuk I, Buttyan R. Apoptosis and expression of Bcl-2, Bcl-XL, and Bax in renal cell carcinomas. *Cancer Invest* 2002, 20: 324–332.

[386] Martin B, Paesmans M, Berghmans T, Branle F, Ghisdal L, Mascaux C, Meert AP, Steels E, Vallot F, Verdebout JM, Lafitte JJ, Sculier JP. Role of Bcl-2 as a prognostic factor for survival in lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2003, 89: 55–64.

[387] Fontanini G, Vignati S, Bigini D, Mussi A, Lucchi M, Angeletti CA, Basolo F, Bevilacqua G. Bcl-2 protein: a prognostic factor inversely correlated to p53 in non small cell lung cancer. *Br J Cancer* 1995; 71: 1003–1007.

[388] Kamesaki S, Kamesaki H, Jorgensen TJ, Tanizawa A, Pommier Y, Cossman J. bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. *Cancer Res* 1993; 53: 4251–4256.

[389] Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, Guyotat D. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* 1993; 81: 3091–3096.

[390] Karakas T, Maurer U, Weidmann E, Miething CC, Hoelzer D, Bergmann L. High expression of bcl-2 mRNA as a determinant of poor prognosis in acute myeloid leukemia. *Ann Oncol* 1998; 9: 159–165.

[391] Barrey O, Zimra Y, Shaklai M, Okon E, Rabizadeh E. Bcl-2, Bcl-X, Bax, and Bak expression in short- and long lived patients with diffuse large B-cell lymphomas. *Clin Cancer Res* 1999, 5: 2860–2866.

[392] Tothova E, Fricova M, Stecova N, Kafkova A, Elbertova A. High expression of Bcl 2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Neoplasma* 2002; 49: 141–144.

[393] Fontana JA, Sun RJ, Rishi AK, Dawson MI, Ordonez JV, Zhang Y, Tschang SH, Bhalla K, Han Z, Wyche J, Poirer G, Sheikh MS, Shroot B, Reichert U. Overexpression of bcl 2 or bcl XL fails to inhibit apoptosis mediated by a novel retinoid. *Oncol Res* 1998; 10: 313–324.

[394] Murata M, Nagai M, Fujita M, Ohmori M, Takahara J. Calphostin C synergistically induces apoptosis with VP-16 in lymphoma cells which express abundant phosphorylated Bcl-2 protein. *Cell Mol Life Sci* 1997; 53: 737–743.

[395] Deng X, Kornblau SM, Ruvolo PP, May WS Jr. Regulation of Bcl2 phosphorylation and potential significance for leukemic cell chemoresistance. *J Natl Cancer Inst Monogr* 2001; 28: 30–37.

[396] Riordan FA, Foroni L, Hoffbrand AV, Mehta AB, Wickremasinghe RG. Okadaic acid-induced apoptosis of HL60 leukemia cells is preceded by destabilization of bcl-2 mRNA and downregulation of bcl-2 protein. *FEBS Lett* 1998; 435: 195–198.

[397] Chi KC, Wallis AE, Lee CH, De Menezes DL, Sartor J, Dragowska WH, Mayer LD. Effects of Bcl-2 modulation with G3139 antisense oligonucleotide on human breast cancer cells are independent of inherent Bcl 2 protein expression. *Breast Cancer Res Treat* 2000; 63: 199–212.

[398] Waters JS, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F, Cotter FE. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non Hodgkin's lymphoma. *J Clin Oncol* 2000; 18: 1812–1823.

[399] Klasa RJ, Gillum AM, Klem RE, Frankel SR. Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense Nucleic Acid Drug Dev* 2002, 12: 193–213.

[400] Wang JL, Zhang ZJ, Choksi S, Shan S, Lu Z, Croce CM, Alnemri ES, Korngold R, Huang Z. Cell permeable Bcl-2 binding peptides: a chemical approach to apoptosis induction in tumor cells. *Cancer Res* 2000; 60: 1498–1502.

[401] Biroccio A, Leonetti C, Zupi G. The future of antisense therapy: combination with anticancer treatments. *Oncogene* 2003; 22: 6579–6588.

[402] MacCarthy-Morrogh L, Wood L, Brimmell M, Johnson PW, Packham G. Identification of a novel human BCL-X promoter and exon. *Oncogene* 2000, 19: 5534–5538.

[403] Watanabe J, Kushihata F, Honda K, Mominoki K, Matsuda S, Kobayashi N. Bcl xL overexpression in human hepatocellular carcinoma. *Int J Oncol* 2002; 21: 515–519.

[404] Gobe G, Rubin M, Williams G, Sawczuk I, Buttyan R. Apoptosis and expression of Bcl-2, Bcl-XL, and Bax in renal cell carcinomas. *Cancer Invest* 2002; 20: 324–332.

[405] Nicot C, Mahieux R, Takemoto S, Franchini G. Bcl X(L) is up regulated by HTLV I and HTLV II in vitro and in ex vivo ATLL samples. *Blood* 2000; 96: 275–281.

[406] Biroccio A, Benassi B, D'Agnano I, D'Angelo C, Buglioni S, Mottolese M, Ricciotti A, Citro G, Cosimelli M, Ramsay RG, Calabretta B, Zupi G. c-Myb and Bcl-x overexpression predicts poor

prognosis in colorectal cancer: clinical and experimental findings. *Am J Pathol* 2001; 158: 1289–1299.

[407] Jaattela M, Benedict M, Tewari M, Shayman JA, Dixit VM. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene* 1995; 10: 2297–2305.

[408] Hattori T, Ookawa N, Fujita R, Fukuchi K. Heterodimerization of Bcl-2 and Bcl-X(L) with Bax and Bad in colorectal cancer. *Acta Oncol* 2000; 39: 495–500.

[409] Kitada S, Krajewska M, Zhang X, Scudiero D, Zapata JM, Wang HG, Shabaik A, Tudor G, Krajewski S, Myers TG, Johnson GS, Sausville EA, Reed JC. Expression and location of pro-apoptotic Bcl-2 family protein BAD in normal human tissues and tumor cell lines. *Am J Pathol* 1998; 152: 51–61.

[410] Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 1996; 87: 619–628.

[411] Ranger AM, Zha J, Harada H, Datta SR, Danial NN, Gilmore AP, Kutok JL, Le Beau MM, Greenberg ME, Korsmeyer SJ. Bad deficient mice develop diffuse large B cell lymphoma. *Proc Natl Acad Sci USA* 2003; 100: 9324–9329.

[412] Zinkel SS, Ong CC, Ferguson DO, Iwasaki H, Akashi K, Bronson RT, Kutok JL, Alt FW, Korsmeyer SJ. Proapoptotic BID is required for myeloid homeostasis and tumor suppression. *Genes Dev* 2003; 17: 229–239.

[413] Wang X, Ryter SW, Dai C, Tang ZL, Watkins SC, Yin XM, Song R, Choi AM. Necrotic cell death in response to oxidant stress involves the activation of the apoptogenic caspase-8/bid pathway. *J Biol Chem* 2003; 278: 29184–29191.

[414] Wagner KW, Engels IH, Deveraux QL. Caspase 2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. *J Biol Chem* 2004; 279: 35047–35052.

[415] Werner AB, Tait SW, de Vries E, Eldering E, Borst J. Requirement for aspartate-cleaved bid in apoptosis signalling by DNA-damaging anti-cancer regimens. *J Biol Chem* 2004; 279: 28771–28780.

[416] Knudson CM, Johnson GM, Lin Y, Korsmeyer SJ. Bax accelerates tumorigenesis in p53-deficient mice. *Cancer Res* 2001; 61: 659–665.

[417] Kaneuchi M, Yamashita T, Shindoh M, Segawa K, Takahashi S, Furuta I, Fujimoto S, Fujinaga K. Induction of apoptosis by the p53-273L (Arg → Leu) mutant in HSC3 cells without transactivation of p21Waf1/Cip1/Sdi1 and bax. *Mol Carcinog* 1999; 26: 44–52.

[418] Sturm I, Papadopoulos S, Hillebrand T, Benter T, Luck HJ, Wolff G, Dorken B, Daniel PT. Impaired BAX protein expression in breast cancer: mutational analysis of the BAX and the p53 gene. *Int J Cancer* 2000; 87: 517–521.

[419] Salah-eldin A, Inoue S, Tsuda M, Matsuura A. Abnormal intracellular localization of Bax with a normal membrane anchor domain in human lung cancer cell lines. *Jpn J Cancer Res* 2000; 91: 1269–1277.

[420] Inoue K, Kohno T, Takakura S, Hayashi Y, Mizoguchi H, Yokota J. Frequent microsatellite instability and BAX mutations in T cell acute lymphoblastic leukemia cell lines. *Leuk Res* 2000; 24: 255–262.

[421] Marone M, Ferrandina G, Macchia G, Mozzetti S, de Pasqua A, Benedetti-Panici P, Mancuso S, Scambia G. Bcl-2, Bax, Bcl-x(L) and Bcl-x(S) expression in neoplastic and normal endometrium. *Oncology* 2000; 58: 161–168.

[422] Gil J, Yamamoto H, Zapata JM, Reed JC, Perucho M. Impairment of the proapoptotic activity of Bax by missense mutations found in gastrointestinal cancers. *Cancer Res* 1999; 59: 2034–2037.

[423] McJilton MA, Van Sikes C, Wescott GG, Wu D, Foreman TL, Gregory CW, Weidner DA, Harris Ford O, Morgan Lasater A, Mohler JL, Terrian DM. Protein kinase Cepsilon interacts with Bax and promotes survival of human prostate cancer cells. *Oncogene* 2003; 22: 7958–7968.

[424] Lu Y, Yagi T. Apoptosis of human tumor cells by chemotherapeutic anthracyclines is enhanced by Bax overexpression. *J Radiat Res (Tokyo)* 1999; 40: 263–272.

[425] Shinoura N, Saito K, Yoshida Y, Hashimoto M, Asai A, Kirino T, Hamada H. Adenovirus-mediated transfer of bax with caspase-8 controlled by myelin basic protein promoter exerts an enhanced cytotoxic effect in gliomas. *Cancer Gene Ther* 2000; 7: 739–748.

[426] Xiang J, Gomez-Navarro J, Arafat W, Liu B, Barker SD, Alvarez RD, Siegal GP, Curiel DT. Pro-apoptotic treatment with an adenovirus encoding Bax enhances the effect of chemotherapy in ovarian cancer. *J Gene Med* 2000; 2: 97–106.

[427] Li X, Marani M, Yu J, Nan B, Roth JA, Kagawa S, Fang B, Denner L, Marcelli M. Adenovirus-mediated Bax overexpression for the induction of therapeutic apoptosis in prostate cancer. *Cancer Res* 2001; 61: 186–191.

[428] Sawai H, Kawai S, Domae N. Reduced expression of Bax in ceramide-resistant HL-60 subline. *Biochem Biophys Res Commun* 2004; 319: 46–49.

[429] Tong QS, Zheng LD, Wang L, Liu J, Qian W. BAK overexpression mediates p53-independent apoptosis inducing effects on human gastric cancer cells. *BMC Cancer* 2004; 4: 33.

[430] Kondo S, Shinomura Y, Miyazaki Y, Kiyohara T, Tsutsui S, Kitamura S, Nagasawa Y, Nakahara M, Kanayama S, Matsuzawa Y. Mutations of the bak gene in human gastric and colorectal cancers. *Cancer Res* 2000; 60: 4328–4330.

[431] Jackson S, Harwood C, Thomas M, Banks L, Storey A. Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. *Genes Dev* 2000; 14: 3065–3073.

[432] Pataer A, Fang B, Yu R, Kagawa S, Hunt KK, McDonnell TJ, Roth JA, Swisher SG. Adenoviral Bak overexpression mediates caspase-dependent tumor killing. *Cancer Res* 2000; 60: 788–792.

[433] Herberg JA, Phillips S, Beck S, Jones T, Sheer D, Wu JJ, Prochazka V, Barr PJ, Kiefer MC, Trowsdale J. Genomic structure and domain organisation of the human Bak gene. *Gene* 1998; 211: 87–94.

[434] Kersemaekers AM, Kenter GG, Hermans J, Fleuren GJ, van de Vijver MJ. Allelic loss and prognosis in carcinoma of the uterine cervix. *Int J Cancer* 1998; 79: 411–417.

[435] Nair P, Nair KM, Jayaprakash PG, Pillai MR. Decreased programmed cell death in the uterine cervix associated with high risk human papillomavirus infection. *Pathol Oncol Res* 1999; 5: 95–103.

[436] Krajewski S, Krajewska M, Reed JC. Immunohistochemical analysis of in vivo patterns of Bak expression, a proapoptotic member of the Bcl-2 protein family. *Cancer Res* 1996; 56: 2849–2855.

[437] Wani KM, Huilgol NG, Hongyo T, Shah K, Chatterjee N, Nair CK, Nomura T. Genetic alterations in the coding region of the bak gene in uterine cervical carcinoma. *Br J Cancer* 2003; 88: 1584–1586.

[438] Wang SH, Mezosi E, Wolf JM, Cao Z, Utsugi S, Gauger PG, Doherty GM, Baker JR Jr. IFNgamma sensitization to TRAIL-induced apoptosis in human thyroid carcinoma cells by upregulating Bak expression. *Oncogene* 2004; 23: 928–935.

[439] Cartron PF, Juin P, Oliver L, Martin S, Meflah K, Vallette FM. Nonredundant role of Bax and Bak in Bid-mediated apoptosis. *Mol Cell Biol* 2003; 23: 4701–4712.

[440] Wilson JW, Nostro MC, Balzi M, Faraoni P, Cianchi F, Becciolini A, Potten CS. Bcl-w expression in colorectal adenocarcinoma. *Br J Cancer* 2000; 82: 178–185.

[441] Lee HW, Lee SS, Lee SJ, Um HD. Bcl-w is expressed in a majority of infiltrative gastric adenocarcinomas and suppresses the cancer cell death by blocking stress-activated protein kinase/c-Jun NH2-terminal kinase activation. *Cancer Res* 2003; 63: 1093–1100.

[442] Pritchard DM, Print C, O'Reilly L, Adams JM, Potten CS, Hickman JA. Bcl-w is an important determinant of damage-induced apoptosis in epithelia of small and large intestine. *Oncogene* 2000; 19: 3955–3959.

[443] Spets H, Stromberg T, Georgii-Hemming P, Siljason J, Nilsson K, Jernberg-Wiklund H. Expression of the bcl-2 family of pro- and anti-apoptotic genes in multiple myeloma and normal plasma cells: regulation during interleukin-6(IL-6)-induced growth and survival. *Eur J Haematol* 2002; 69: 76–89.

[444] Lee SH, Soung YH, Lee JW, Kim HS, Lee JH, Kim HS, Lee JH, Park JY, Cho YG, Kim CJ, Kim SY, Park WS, Kim SH, Lee JY, Yoo NJ. Mutational analysis of Noxa gene in human cancers. *APMIS* 2003; 111: 599–604.

[445] Jansson AK, Emterling AM, Arbman G, Sun XF. Noxa in colorectal cancer: a study on DNA, mRNA and protein expression. *Oncogene* 2003; 22: 4675–4678.

[446] Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci USA* 2003; 100: 1931–1936.

[447] Jansson A, Arbman G, Sun XF. mRNA and protein expression of PUMA in sporadic colorectal cancer. *Oncol Rep* 2004; 12: 1245–1249.

[448] Sanchez-Carbayo M, Urrutia M, Gonzalez de Buitrago JM, Navajo JA. Evaluation of two new urinary tumor markers: bladder tumor fibronectin and cytokeratin 18 for the diagnosis of bladder cancer. *Clin Cancer Res* 2000; 6: 3585–3594.

[449] Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. *Dev Cell* 2001; 1: 645–653.

[450] Egle A, Harris AW, Bouillet P, Cory S. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci USA* 2004; 101: 6164–6169.

[451] Jiang Z, Zheng X, Lytle RA, Higashikubo R, Rich KM. Lovastatin-induced up-regulation of the BH3-only protein, Bim, and cell death in glioblastoma cells. *J Neurochem* 2004; 89: 168–178.

[452] Latil A, Bieche I, Chene L, Laurendeau I, Berthon P, Cussenot O, Vidaud M. Gene expression profiling in clinically localised prostate cancer: a four-gene expression model predicts clinical behavior. *Clin Cancer Res* 2003; 9: 5477–5485.

[453] Yasui K, Mihara S, Zhao C, Okamoto H, Saito-Ohara F, Tomida A, Funato T, Yokomizo A, Naito S, Imoto I, Tsuruo T, Inazawa J. Alteration in copy numbers of genes as a mechanism for acquired drug resistance. *Cancer Res* 2004; 64: 1403–1410.

[454] Kim JK, Kim KD, Lee E, Lim JS, Cho HJ, Yoon HK, Cho MY, Baek KE, Park YP, Paik SG, Choe YK, Lee HG. Up-regulation of Bfl-1/A1 via NF-kappaB activation in cisplatin-resistant human bladder cancer cell line. *Cancer Lett* 2004; 212: 61–70.

[455] Tarte K, Jourdan M, Veyrune JL, Berberich I, Fiol G, Redal N, Shaughnessy J Jr, Klein B. The Bcl-2 family member Bfl-1/A1 is strongly repressed in normal and malignant plasma cells but is a potent anti-apoptotic factor for myeloma cells. *Br J Haematol* 2004; 125: 373–382.

[456] Sanz L, Garcia-Marco JA, Casanova B, de La Fuente MT, Garcia-Gila M, Garcia-Pardo A, Silva A. Bcl-2 family gene modulation during spontaneous apoptosis of B-chronic lymphocytic leukemia cells. *Biochem Biophys Res Commun* 2004; 315: 562–567.

[457] D'Souza B, Rowe M, Walls D. The bfl-1 gene is transcriptionally upregulated by the Epstein-Barr virus LMP1, and its expression promotes the survival of a Burkitt's lymphoma cell line. *J Virol* 2000; 74: 6652–6658.

[458] D'Sa-Eipper C, Subramanian T, Chinnadurai G. bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. *Cancer Res* 1996; 56: 3879–3882.

[459] Yoon HS, Hong SH, Kang HJ, Ko BK, Ahn SH, Huh JR. Bfl-1 gene expression in breast cancer: its relationship with other prognostic factors. *J Korean Med Sci* 2003; 18: 225–230.

[460] Talieri M, Diamandis EP, Katsaros N, Gourgiotis D, Scorilas A. Expression of BCL2L12, a new member of apoptosis-related genes, in breast tumors. *Thromb Haemost* 2003, 89: 1081–1088.

[461] Mathioudaki K, Scorilas A, Papadokostopoulou A, Xynopoulos D, Arnogianaki N, Agnanti N, Talieri M. Expression analysis of BCL2L12, a new member of apoptosis-related genes, in colon cancer. *Biol Chem* 2004; 385: 779–783.

[462] Floros KV, Thomadaki H, Lallas G, Katsaros N, Talieri M, Scorilas A. Cisplatin-induced apoptosis in HL-60 human promyelocytic leukemia cells: differential expression of BCL2 and novel apoptosis-related gene BCL2L12. *Ann NY Acad Sci* 2003; 1010: 153–158.

[463] Floros KV, Thomadaki H, Katsaros N, Talieri M, Scorilas A. mRNA expression analysis of a variety of apoptosis-related genes, including the novel gene of the BCL2-family, BCL2L12, in HL-60 leukemia cells after treatment with carboplatin and doxorubicin. *Biol Chem* 2004; 385: 1099–1103.

[464] Castells A, Ino Y, Louis DN, Ramesh V, Gusella JF, Rustgi AK. Mapping of a target region of allelic loss to a 0.5 cM interval on chromosome 22q13 in human colorectal cancer. *Gastroenterology* 1999; 117: 831–837.

[465] Daniel PT, Pun KT, Ritschel S, Sturm I, Holler J, Dorken B, Brown R. Expression of the death gene Bik/Nbk promotes sensitivity to drug-induced apoptosis in corticosteroid-resistant T-cell lymphoma and prevents tumor growth in severe combined immunodeficient mice. *Blood* 1999; 94: 1100–1107.

[466] Zou Y, Peng H, Zhou B, Wen Y, Wang SC, Tsai EM, Hung MC. Systemic tumor suppression by the proapoptotic gene bik. *Cancer Res* 2002; 62: 8–12.

[467] Li YM, Wen Y, Zhou BP, Kuo HP, Ding Q, Hung MC. Enhancement of Bik antitumor effect by Bik mutants. *Cancer Res* 2003; 63: 7630–7633.

[468] Cho-Vega JH, Rassidakis GZ, Admirand JH, Oyarzo M, Ramalingam P, Paraguya A, McDonnell TJ, Amin HM, Medeiros LJ. MCL-1 expression in B-cell non-Hodgkin's lymphomas. *Hum Pathol* 2004; 35: 1095–1100.

[469] Backus HH, van Riel JM, van Groeningen CJ, Vos W, Dukers DF, Bloemena E, Wouters D, Pinedo HM, Peters GJ. Rb, mcl-1 and p53 expression correlate with clinical outcome in patients with liver metastases from colorectal cancer. *Ann Oncol* 2001; 12: 779–785.

[470] Tang L, Tron VA, Reed JC, Mah KJ, Krajewska M, Li G, Zhou X, Ho VC, Trotter MJ. Expression of apoptosis regulators in cutaneous malignant melanoma. *Clin Cancer Res* 1998; 4: 1865–1871.

[471] Shigemasa K, Katoh O, Shiroyama Y, Mihara S, Mukai K, Nagai N, Ohama K. Increased MCL-1 expression is associated with poor prognosis in ovarian carcinomas. *Jpn J Cancer Res* 2002; 93: 542–550.

[472] Wakabayashi K, Saito H, Kaneko F, Nakamoto N, Tada S, Hibi T. Gene expression associated with the decrease in malignant phenotype of human liver cancer cells following stimulation with a histone deacetylase inhibitor. *Int J Oncol* 2005; 26: 233–239.

[473] Fong PY, Xue WC, Ngan HY, Chan KY, Khoo US, Tsao SW, Chiu PM, Man LS, Cheung AN. Mcl 1 expression in gestational trophoblastic disease correlates with clinical outcome: a differential expression study. *Cancer* 2005; 103: 268–276.

[474] O'Driscoll L, Cronin D, Kennedy SM, Purcell R, Linehan R, Glynn S, Larkin A, Scanlon K, McDermott EW, Hill AD, O'Higgins NJ, Parkinson M, Clynes M. Expression and prognostic relevance of Mcl 1 in breast cancer. *Anticancer Res* 2004; 24: 473–482.

[475] Spets H, Stromberg T, Georgii-Hemming P, Siljason J, Nilsson K, Jernberg-Wiklund H. Expression of the bcl 2 family of pro and anti apoptotic genes in multiple myeloma and normal plasma cells: regulation during interleukin-6 (IL-6)-induced growth and survival. *Eur J Haematol* 2002; 69: 76–89.

[476] Ma Y, Cress WD, Haura EB. Flavopiridol-induced apoptosis is mediated through up-regulation of E2F1 and repression of Mcl 1. *Mol Cancer Ther* 2003; 2: 73–81.

[477] Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* 1992; 44: 1–80.

[478] Scorilas A, Yotis J, Gouriotis D, Keramopoulos A, Ampela K, Trangas T, Talieri M. Cathepsin-D and c-erb-B 2 have an additive prognostic value for breast cancer patients. *Anticancer Res* 1993; 13: 1895–1900.

[479] Ardavanis A, Gerakini F, Amanatidou A, Scorilas A, Pateras C, Garoufali A, Pissakas G, Stravolemos K, Apostolikas N, Yiotis I. Relationships between cathepsin D, pS2 protein and hormonal receptors in breast cancer cytosols: inconsistency with their established prognostic significance. *Anticancer Res* 1997; 17: 3665–3669.

[480] Ardavanis A, Scorilas A, Loukeri A, Gerakini F, Pissakas G, Missitzis I, Apostolikas N, Yiotis I. Cathepsin D may help in discriminating node-negative breast cancer patients at risk for local-regional recurrence. *Anticancer Res* 1998; 18: 2885–2890.

[481] Scorilas A, Yotis J, Pateras C, Trangas T, Talieri M. Predictive value of c-erbB 2 and cathepsin-D for Greek breast cancer patients using univariate and multivariate analysis. *Clin Cancer Res* 1999; 5: 815–821.

[482] Scorilas A, Trangas T, Yotis J, Pateras C, Talieri M. Determination of c-myc amplification and overexpression in breast cancer patients: evaluation of its prognostic value against c-erbB-2, cathepsin D and clinicopathological characteristics using univariate and multivariate analysis. *Br J Cancer* 1999; 81: 1385–1391.

[483] Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR, Thompson CB. The combined functions of proapoptotic Bcl 2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 2000; 6: 1389–1399.

[484] Talieri M, Papadopoulou S, Scorilas A, Xynopoulos D, Arnogianaki N, Plataniotis G, Yotis J, Agnanti N. Cathepsin B and cathepsin D expression in the progression of colorectal adenoma to carcinoma. *Cancer Lett* 2004; 205: 97–106.

EXHIBIT B

ORIGINAL RESEARCH

Enhancement of radiation sensitivity with BH3I-1 in non-small cell lung cancer

Roa W[a]
Chen H[c]
Alexander A[a]
Gulavita S[b]
Th'ng J[b]
Sun XJ[c]
Petruk K[d]
Moore R[d]

From: Departments of Radiation Oncology[a], Experimental Oncology[c], and Department of Surgery[d], Cross Cancer Institute, Edmonton, Alberta, Canada. Northwestern Ontario Regional Cancer Centre[b], Thunder Bay, Ontario, Canada

Submitted: 9th November 2004;
Accepted for publication 17th February 2005

Clin Invest Med 2005; 28 (2): 55–63

Abstract

Background: Anti apoptotic proteins, such as Bcl-2 and Bcl-x_L, are frequently over expressed in human malignancies, and this is correlated with resistance to chemotherapeutic drugs and γ- radiation. Recently identified small organic molecules capable of inhibiting Bcl 2 and/or Bcl-x_L function, may enhance radiation sensitivity of cancer cells in which they are over expressed. We examined whether specific blockade of the BH3 domain binding to Bcl-x_L could sensitize cancer cells to γ- radiation.
Methods: Human non-small-cell lung cancer H460 cells with wild type p53 and H1792 cells with mutant p53 were exposed to various doses of radiation and/or BH3I 1 and for different points of time to BH3I 1 treatment. XTT and clonogenic survival assays were used to evaluate the growth inhibitory effects of the antagonist BH3I-1, ionizing radiation or both. Western blot analysis was used to examine the cellular effect of the expression of Bcl x_L, Bax, and p53. Apoptosis and cell cycle distribution were analyzed by confocal microscopy with Hoechst 33258 staining and cytochrome *c*, and flow cytometry, respectively.
Results: BH3I 1 appeared to induce a dose- and time-dependent apoptosis in H460 and H1792 cells, regardless of p53 status. After 2 days of BH3I 1 treatment, the cells that remained attached were exposed to ionizing radiation. Followed by clonogenic assay, BH3I-1 treatment enhanced the radiation sensitivity of H1792 surviving cells with mutant p53, but not in H460 cells with wild type p53. A transient time dependent cell cycle blockade at G_2 M phase was identified for H1792 cells without subsequent modification of cell cycle distribution.
Conclusion: These findings suggest a potential role for the small molecule inhibitor as a novel radiation sensitizer in non small cell lung cancer.

Lung cancer is the leading cause of cancer death in North America and throughout the world.[1] Despite significant advances in appreciation of the underlying molecular mechanisms, current treatments including surgery, conventional chemotherapy and radiotherapy, confers limited benefit. The long-term survival rate for lung cancer patients remains very poor, with 15% or less surviving five years.[1]

The anti-apoptotic proteins Bcl-2 and Bcl-x_L are over expressed in many human cancers including lung cancer[2-4], rendering cancer resistance to a wide spectrum of chemo-and radiotherapy.[5,6] Because the cellular commitment to apoptosis is regulated by competitive heterodimerization between pairs of pro-survival proteins such as Bcl-2 and Bcl-x_L, and pro-apoptotic proteins such as Bax and Bak[7-9], the therapeutic ratio may be improved if the balance of

Bax/Bcl 2 or Bax/Bcl x_L heterodimerization is tilted in favor of cell death. As such, anti sense oligonucleotides inhibiting Bcl-2 or Bcl-x_L expression have been shown to be inducers of apoptosis and sensitize cancer cells to chemotherapy.[10-12] Other studies have shown that human Bax-BH3 and Bak BH3 peptides disrupt Bax/Bcl-2 and Bax/Bcl-x_L heterodimerizations and can induce apoptosis in various cell types.[13,14] Moreover, a Bax BH3 peptide has been found to induce release of cytochrome c from the mitochondria, but failed to overcome the protective effects of Bcl-2 over-expression.[15]

In recent years, non-peptidic, cell-permeable, small molecule inhibitors that bind to the BH3 binding sites of Bcl-2 and Bcl-x_L have been identified by computerized, high throughput screening assays using a library of more than 20,000 chemicals from the Chemical Directory.[16-19] These compounds induce apoptosis, inhibit cell growth by triggering the release of cytochrome c through activation of caspases. These small molecules thus represent a novel class of anti-cancer therapies, which promote apoptosis of resistant cancer cells.

Although Bcl-2 and its related protein Bcl-x_L possess important anti apoptotic function in a variety of cancers, their relevance in conferring radiation resistance remains undefined.[20] A recent study indicated that Bcl 2 family protein expression can modulate radiosensitivity in human glioma cells[21], and that Bcl x_L protects glioma cells from apoptosis by blocking ionizing radiation-induced release of cytochrome c.[22] Currently, few data are available on the impact of Bcl x_L activity with small molecule antagonists in irradiated lung cancer cells. Thus, antagonists that specifically target the anti-apoptotic function of Bcl-x_L may have clinical utility by its enhancing radiation sensitivity of cancer cells.

In this study, we examined the effect of a BH3 mimetic small molecule antagonist, BH3I-1, which has been shown to induce apoptosis by disrupting protein protein interaction of the BH3-domain between pairs of pro-survival and pro apoptotic Bcl 2 family members in Non Small-Cell Lung Cancer (NSCLC).[17] Here we report that BH3I-1 can lead to the release of cytochrome c, induce apoptosis regardless of p53 status, and enhance radiation sensitivity of NSCLC cells with mutant p53, in correspondence to a G_2 M phase cell cycle arrest.

Materials and Methods

Cell Culture and Reagents

Human NSCLC H460 cells with wild-type p53, and H1792 cells with mutant p53[23,24], were obtained from American Type Culture Collection (ATCC, Rockville MD). They were maintained in RPMI 1640 (Gibco BRL), with 10% fetal calf serum and 1% antibiotics in a humidified atmosphere of 5% CO_2. Cell proliferating kit (XTT) was purchased from Roche Molecular Biochemical (Laval, Quebec, Canada). Rabbit polyclonal antibodies against extra-cellular signal-regulated kinase 1 and 2 (ERK1/2) were purchased from Stress Biotechnology Corp. (Victoria, BC, Canada). Rabbit polyclonal antibodies against p53 and the peroxidase-conjugated goat anti mouse and anti rabbit antibodies were from Cell Signaling Technology (Beverly, MA). BH3I-1[5-(p-Bromobenzylidine)-a-isopropyl-4-oxo-2-thioxo 3 thiozolidineacetic acid], Mouse monoclonal anti-Bcl-x_L and anti-Bax antibodies were from Calbiochem-Novabiochem International (San Diego, CA). Mouse monoclonal anti-cytochrome c was obtained from Chemicon International, Inc. (Temecular, CA). Hoechst 33258, propidium iodide and RNase A were purchased from Sigma Chemical Incor. (St. Louis, MO)

Cell Viability Assay

The dose-dependent effect of BH3I 1 on the viability of NSCLC was assessed by using a colorimetric XTT assay according to manufacturer's instructions from Roche Molecular Biochemicals (Laval, QC). In brief, cell suspension containing $1x10^4$ cells in 100uL of medium per well were seeded in 96-well flat bottom microplates, allowed to adhere overnight and then incubated with BH3I 1 at different concentrations for 48 h alone or followed by irradiation with graded doses (2 Gy, 8Gy) using a 60Co irradiator at ~1.2 Gy/min at room temperature. After 48 h, XTT labeling mixture (50μL/well) was added to each well and incubated for 4h at 37°C. Plates were read at 490 nm with a reference wavelength of 650 nm using the Benchmark microplate reader (BioRad Laboratory, Mississauga, ON.). The percentage of viable cells was calculated as follows: 1-(optical density of treated cells/optical density of untreated cell control) x100. All of the assays were performed at least twice in triplicate. The concentration of agents required for IC 50 was calculated as that giving a 50% decrease in absorbance compared with controls incubated simultaneously without agents.

Clonogenic Survival Assay

Cells were seeded at appropriate densities in triplicate to yield 50-200 colonies/well in 6-well plates (60mm), and were counted by manual cell counts of live cells (excluding floating cells) as assessed with Trypan blue exclusion. For the drug clonogenic assay, tumour cells were exposed to different concentrations of BH3I-1 as indicated in the figure legends or vehicle (0.1% DMSO) for 1 or 2 days. Floating cells were collected for further apoptotic analysis and the remaining attached cells were removed from the plates by trypsin/EDTA, and replated in specified numbers onto 6-well plates in drug-free media for determination of clonogenic ability (750cells/well). For this clonogenic assay, the floating cells (those that underwent apoptosis) were ignored, and only the attached cells after 48 hours of drug treatment were utilized in the analysis of clonogenic ability.

For the radiation clonogenic assay, BH3I-1 pretreated tumour cells (2 days before radiation) were either mock irradiated or irradiated with graded doses (2-8Gy) radiation using a ^{60}Co irradiator at 1.2 Gy/min. Trypsinized cells were replated onto 6 well plates for determination of colony-forming ability. The plates were incubated for 8-14 days depending on cell doubling time and re-fed every 3-4 days to allow clonogenic growth. Colonies were washed in cold PBS, fixed in cold acetone/methanol (1:1) for 10 min, stained with 1% methylene Blue in PBS for 15 min and de-stained with running tap water and air dry. Colonies that contained more than 50 but less than 200 cells were scored. Radiation survival curves were constructed after normalization for the cytotoxicity induced by the BH3I-1 alone, and were calculated as the mean of colonies counted divided by the number of cells plated times the plating efficiency. Plating efficiency was the mean of colonies divided by the number of cells inoculated without radiation. Clonogenic survival was plotted as a function of dose on a semi-logarithmic plot. Each data point represented the mean results of three wells from each of the two independent experiments.

Fluorescence Immunocytochemistry

Cells grown on 8 well chamber glass slides were incubated with 100uM BH3I-1 for 24h, washed in PBS, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After the PBS rinse, the cells were stained with primary mouse anti cytochrome c (1:100 dilution in 0.5% BSA in PBS) or mouse anti-Bax (clone 6A7; 1:100 dilution) antibodies for 2 h and subsequently followed by horseradish peroxidase-conjugated secondary antibodies diluted at 1:400 for 45 min. and counter stained in Hoechst 33258 dye. When indicated, apoptosis for cell morphology was determined by incubating cells with Hoechst 33258 at a concentration of 2.5μg/ml for 20 min at 37°C. The chamber slides were rinsed in PBS and overlaid with mounting media 90% glycerol in PBS. Excitation of the stains was performed on a Carl Zeiss Laser Scanning confocal Microscope (LSM510) mounted on an Axiovert 100M microscope equipped with UV and visible laser lines and analyzed using the LSM imaging browser and Adobe® Photoshop 7. The excitation and emission wavelengths were 570nm and 590nm for Rhodamine (red), 494nm and 518nm for Fluorescence (green), and 352nm and 461nm for Hoechst 33258, respectively.

Flow Cytometric Analysis of Cell Cycle and DNA Fragmentation

Cells were treated with either vehicle (0.1% DMSO) or 100μM of BH3I-1 for 2 days followed by 8Gy of γ rays using a ^{60}Co source (1.2 Gy/min) and then incubated for 48 hours in the absence of BH3I-1. Cells were also treated with 100uM of BH3I-1 and incubated with various time periods (8, 16, 24 and 48 h). Medium containing floating cells was combined with cells trypsinized from the plates and then centrifuged at 1,000 x g for 5 min. Cells were then washed once in ice-cold PBS and fixed in suspension by rapidly admixing with a Pasteur pipette dispensing 1ml cells into 9ml of 80% ethanol in centrifuge tube on ice and stored at -20°C for several days. Cells were washed in PBS and re suspended in 0.5ml of a DNA staining solution containing 50 μg/ml propidium iodide, 0.2mg/ml RNase A and 0.15% Triton X-100 in PBS. Cell cycle analysis was performed on a Becton Dickinson FACSort flow cytometer using the CELLQuest software provided by the manufacturer. For each sample, at least 10,000 cells were used for each analysis. Data analysis was carried out in ModFitLT® software on cells gated on an FL2 area channel vs. FL2-width channel display to exclude clumped cells and cell debris

Western Blot Analysis

Whole cell lysates were prepared as previously described.25. In brief, cells were scraped from the culture, washed twice in ice-cold PBS, and collected in 1.5 mL Eppendorf tubes and then suspended in 200uL of cold lysis buffer(25mM Tris, pH 7.5,150 mM NaCl,

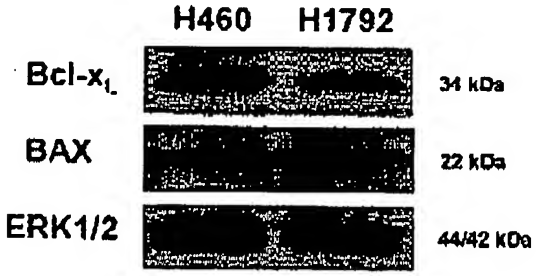

FIGURE 1 Western blot analysis of Bcl-xL and Bax protein in human non small cell lung cancer cells (H460 and H1792). Anti-ERK1/2 antibody is used as a loading control.

1mM EDTA, 1% Triton X-100 and 0.5% NP-40) containing freshly added 4μL/ml of protease inhibitor cocktail and 1mM PMSF for 30 min. on ice. After centrifugation at 2,500 x g for 15 min, the supernatant was retained and the protein concentration was determined with use of Bio-Rad DC kit (Bio-Rad Laboratories, LA) according to the manufacturer's instruction. Aliquots of equal amount of proteins (40-50ug/well) were resolved by SDS-PAGE (10-15%) gels and electroblotted onto nitrocellulose membrane and subjected to Western blot analysis. ERK1/2 are ubiquitous and abundant proteins present in the cytoplasm, and serve as a loading control.

Results

Pro-survival and pro-apoptotic protein expression

Protein expression of Bcl-xL and Bax in the two human NSCLC cell lines H460 and H1792 were evaluated by Western blot analysis. Both lines express Bcl-xl and Bax proteins constitutively as 34kDa and 22kDa bands, respectively (Fig 1). Based on densitometry measurement, H460 cells was found to exhibit a slightly higher level of Bcl-xL than H1792 cells. On the other hand, both cell lines express a similar level of Bax.

Cytotoxicity of BH31-1

BH31-1 induced similar dose-and time dependent cytotoxicity in both cell lines after 48 h incubation (Fig. 2A and B). The IC_{50} of BH31-1 was 100 μM for H460 and H1792 cells as judged by XTT assay. When these cells were treated with 100μM of BH31-1 over a different point of time from 4 to 48 h, >50% of cyto-

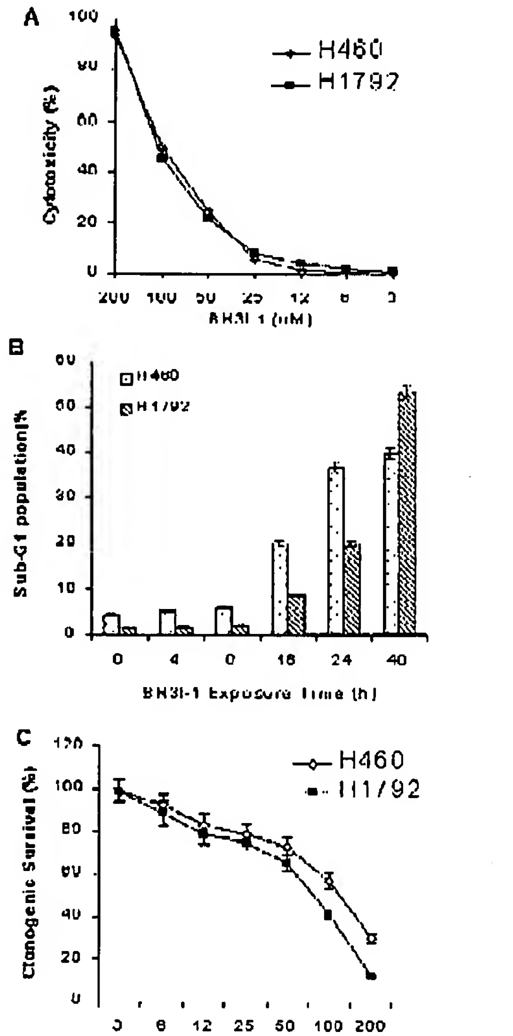

FIGURE 2 Cytotoxicity of BH31-1. (A) H460 and H1792 cells were exposed to various concentrations of BH31-1 for 48 h. Cell viability was analyzed with XTT assay. Percent (%) cytotoxicity was determined by measuring the OD at 490 nm in a microplate reader. (B) H460 and H1792 cells were treated from 4 to 48 h with 100uM BH31-1. Cell cycle distribution was analyzed by fluorescence-activated cell sorting (FACS). Cell cycle phase sub-G1 distribution was determined with a FACScan analyzer. (C) H460 and H1792 cells were exposed to various concentrations of BH31-1 for 48 h. After exposure, attached cells were lifted by trypsinization and re plated for 8 14 days to allow colony forming. Clonogenic survival curves were constructed after normalizing for BH31-1-induced cytotoxicity, with the mean number of untreated control cell colonies set at 100%.

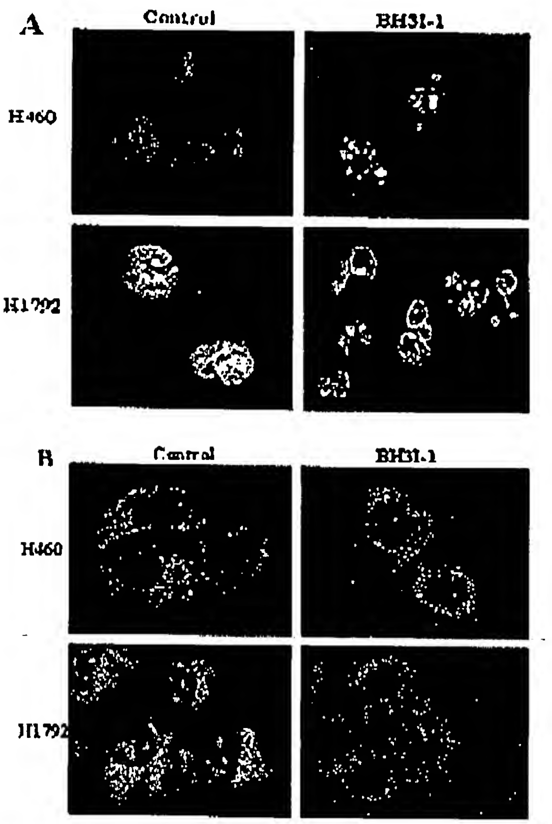

FIGURE 3 BH31-1 induced apoptosis. (A) BH31-1 induced chromatin condensation of cell nuclei stained with Hoechst 33258. Apoptotic cells show strong chromatin condensation and nuclear fragmentation. (B) Confocal microscopy analysis of subcellular location of cytochrome c in untreated and BH31-1 treated cells.

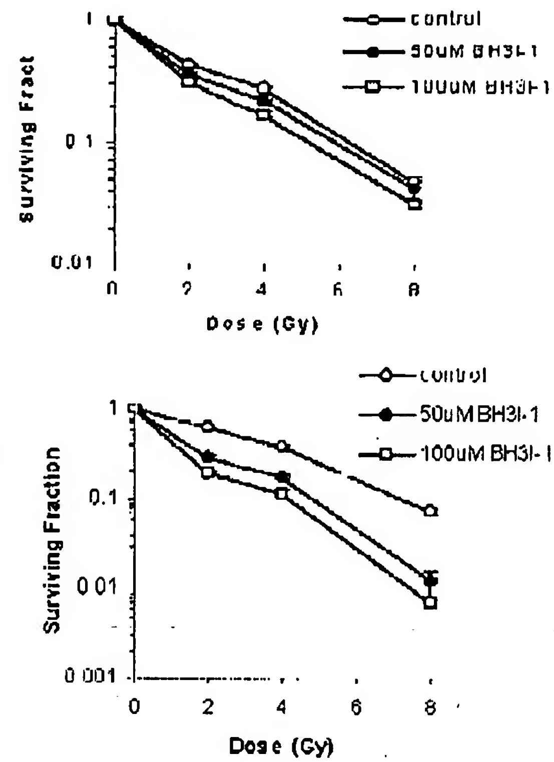

FIGURE 4 Effect of BH31-1 on radiation sensitivity. Cultured cells were either treated in the absence of (o), or presence of 50 µM (•) or 100uM of BH31-1 (□) for 48 h (A and B), followed by graded doses of γ irradiation and plated for colonogenic assay. Similar setup was performed with 100uM of BH31-1 for 1 or 2 days (C and D). The surviving fraction was calculated as the plating efficiency of treated cells/ the plating efficiency of untreated cells, and plotted as a function of dose on a semi logarithmic plot. Each data point represents the means from at least three independent experiments.

toxicity was observed in sub G1 apoptotic phase as determined by a FACScan analyzer. BH31-1 also induced clonogenic cytotoxicity in the tumor cells after 48 hour incubation (Fig. 2C). The IC_{50} of BH31-1 was 100uM and 125uM for H1792 and H460 cells, respectively.

Apoptosis

In order to determine the cytotoxic mechanism of the floating cells that appeared after treatment with BH31-1 alone, nuclear morphology staining by Hoechst 33258 was performed to identify apoptosis (Fig. 3A).

The staining pattern of H460 cells treated with BH31-1 appears as classic apoptosis. The corresponding appearance of H1792 cells treated with BH31-1 shows a similar picture with minor differences, suggestive of a concurrent process additional to apoptosis, which was estimated to be 40%. Under confocal microscopy, BH31-1 treated cells showed a diffuse

FIGURE 5 Influence of p53 expression. Cells were pretreated with 100μM of BH31-1 for 48 h, and then irradiated with graded doses radiation and subjected to Western blot analysis.

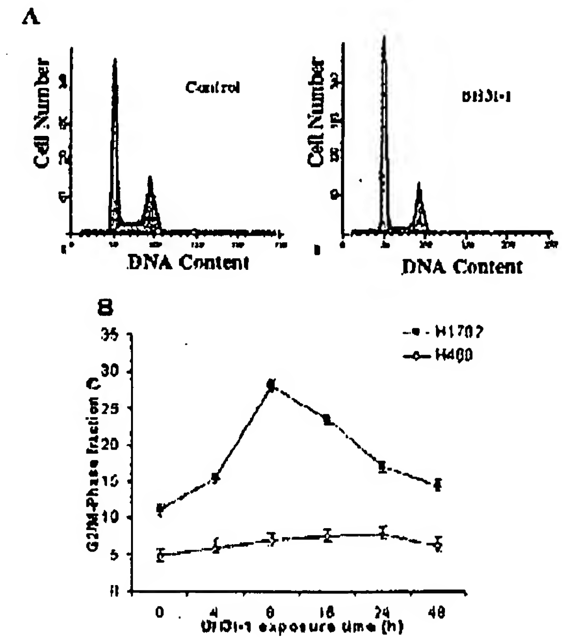

FIGURE 6 BH31-1 effect on cell cycle phase distribution. A, H1792 cells were treated with 100uM of BH31 1 for 48 h prior to FACS analysis for DNA content, showing no significant change. B, Change in G2/M fraction of the cell cycle phase after exposure to 100uM of BH31 1 for various time points as determined by FACS.

immunofluorescence staining pattern of cytochrome c release from the mitochondria. This is in contrast to the control cells that showed a coarse punctate staining pattern (Fig. 3B). Immunohistochemistry study using anti BAX was also performed, and there was no change in its expression before and after drug treatment (data not shown).

Effects of BH31 1 on radiation sensitivity

In order to determine the radiation sensitizing effect of BH31-1, H460 and H1792 cells were exposed to either 50μM or 100μM BH31-1 for 48 h. Surviving cells were then irradiated with graded doses of ?-rays and plated for clonogenic assay. As shown in Fig.4A and B, BH31 1 enhances radiation sensitivity in H1792 cells, where cell killing is increased by more that one log at 8 Gy of irradiation. In contrast, the surviving fraction of the H460 cells is barely affected by BH31 1 at the same dose of 100uM.

P53 expression

The level of expressed p53 after pretreatment with 100uM of BH31 1 for 48 hours and graded doses of ionizing radiation was evaluated by Western Blot. BH31 1 treatment prior to irradiation (lane 0) did not induce p53 expression in H460 cells, but graded irradiation (lanes 2, 4 8) did induce a higher level of expressed p53, as expected in cells with wild type p53 (fig. 5).

BH31-1 effect on cell cycle distribution

In order to determine whether cell-cycle alteration is involved with BH31-1 in H1792 cells, cell-cycle phase distribution was analyzed with flow cytometry. As shown in Fig 6A, no changes in H1792 cell cycle phase distribution are observed at the end of two days of treatment with 100uM of BH31-1 alone. However, an earlier transient arrest of H1792 cells at G2/M phase is identified during the same treatment, starting at 4 h, and settling by 48 h. This was not observed in the H460 cells (Fig. 6B).

Cell cycle distribution after irradiation and BH31-1

The alternation of cell-cycle phase distribution by BH31-1 was further analyzed using flow cytometry in combined with irradiation. When H1792 cells were treated with either irradiation or in combination with BH31-1, the percentage of cells in the G2/M phase of the cell cycle increased from 10% to 20% and 48%, respectively (Fig. 7B). Conversely, the percentage of cells in G1 decreased from 76% to 53% and 35%,

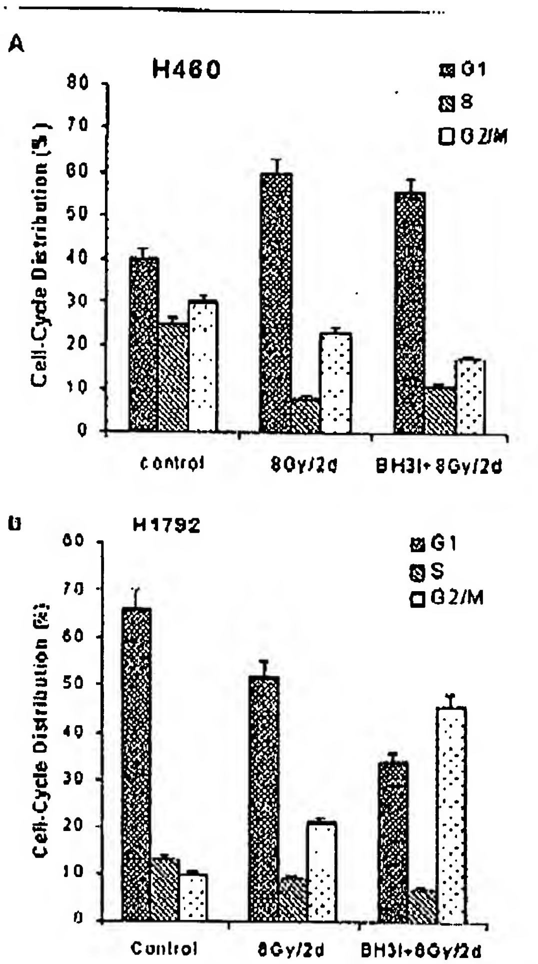

FIGURE 7 The effect of radiation alone, or combined with BH3I 1, on cell cycle distribution. H460 cells (A) and H1792 cells (B) were pre exposed to BH3I-1 for 48h, washed, replenished with culture media, and then irradiated at 8Gy. Cell cycle phase distribution was determined with a FACScan analyzer.

respectively, and S1 phase cells decreased from 13% to 9% and 7%, respectively. This was identified two days after completion of treatment. In contrast, the change was not apparent in H460 cells (Fig. 7A).

Discussion

The BH3 domain plays a crucial role in mediating pro-apoptotic and anti apoptotic activities of Bcl 2 family members such as Bcl x_L and Bax. BH3I 1, a cell-permeable molecule capable of disrupting BH3-domain binding to Bcl-x_L, represents a new generation of small-molecule inhibitor of protein protein interaction. However, there has been little study of BH3I-1 with respect to enhancement of radiation cytotoxicity, in tumour cells. In this study, we have demonstrated with clonogenic assays that BH3I-1 could enhance killing of the human H1792 NSCLC cell line, with release of cytochrome C from the mito chondria, nuclear fragmentation, and apoptosis. Furthermore, H1792 cells were radiosensitized in association with G_2 M arrest. For the surviving H1792 cells (that are left behind after treatment with BH3I-1), there was an increasing radiosensitizing effect with a longer incubation time of BH3I-1, and the cell cycle effect of BH3I 1 might be involved.

It is known that p53 plays important roles in cell cycle regulation and apoptosis.[26,27] We used p53 wild-type H460 and p53 mutant H1792 cells as models to correlate the effects of BH3I-1-induced cytotoxicity. Our data showed that both H460 and H1792 cells were sensitive to the cytotoxic effect of BH3I-1 at similar concentrations, regardless of its p53 status. This suggests that BH3I 1 inhibition of tumor growth may be conveyed via p53-dependent as well as independent pathways. In this study, the staining pattern of the H460 cells with BH3I-1 treatment was characteristic of classical apoptosis, while that of the H1792 cells was atypical of classical apoptosis. This suggests the possibility of a concurrent process, such as necrosis. Indeed, Hao et al.[28] reported that another BH3-mimetic, BH3I 2, was able to induce necrotic cell death in addition to apoptotic induction. Accordingly, the exact mechanisms of BH3I-1 cytotoxicity in the different cell line remain to be further clarified.

Both dose and time interval of the combined BH3I-1 and ionizing radiation could modulate radiation sensitivity in H1792 cells. It is important to note that the concentration of the BH3 mimetic used in this study was higher than has been used in previous studies of such small molecule inhibitors 28 The data suggested that low concentrations of such molecules (<50μM) could result in uncoupling of mitochondrial respiration from oxidative phosphorylation, without cytochrome c release or apoptosis. Higher concentrations, however, would associate with release of cytochrome c, apoptosis, and inhibition of colony formation as noted in this study.[28] Furthermore, although p53 has been implicated in promoting radiation-induced apoptosis[29], the Bcl-x_L protein has been

shown to block radiation-induced apoptosis.[22] A recent report indicated that the expression levels of Bax and Bcl-xL protein in glioblastoma cell lines could modulate their radiosensitivity.21 We speculate that the lack of radiosensitization in H460 cells may be due to the protective effect of Bcl x_L, wherein BH3I 1 is unable to inhibit all Bcl-x_L associated heterodimeriza-tion within the cell. The remaining uninhibited Bcl-x_L protein may then have been sufficient to prevent radiosensitization.

BH3I 1 has been found to induce apoptosis in a variety of leukaemic cell types in a dose and time-dependent manner that correlates with the cytochrome c release from mitochondria.17,19 Our data indicated that a high percentage of H1792 cells underwent apoptotic nuclear fragmentation with release of cytochrome c, and this increased with time after treatment with BH3I-1 Nevertheless, the exact biochemical and molecular processes responsible for the BH3I-1-induced radiosensitization remain to be determined. It has been shown that the p53 status of a cell can affect its inherent radiosensitivity.[30-32] A recent report has suggested that radiation induced apoptosis occurred early in NSCLC cells with wild type p53, whereas radiation induced apoptosis occurred late in mutant or inactive p53 cells. The latter was a downstream secondary event occurring beyond the G2-M phase arrest.[30] In this study we have observed a difference in cell cycle response between H1792 and H160 cells, which may be due to their difference in p53 status. As predicted, the wild type p53 H460 cells exhibited a G1 block in response to BH3I 1 and ionizing radiation. This would allow time to repair DNA damaged cells after irradiation. In contrast, the mutant p53-expressing H1792 cells continued into S phase and G2-M phases after damage from BH3I-1 and radiation.

In conclusion, we have shown that small-molecule Bcl-x_L inhibitor BH3I 1 induces apoptosis in H460 and H1792 cells, regardless of p53 status, and that it enhances the radiosensitivity of the surviving p53 mutant cells to subsequent irradiation. The latter may be related to the induction of a cell cycle block. These findings suggest a role for BH3I-1 as a novel radiation sensitizer in some human NSCLCs.

References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003;53:5-26
2. Pena JC, Thompson CB, Recant W, et al. Bcl-xL and Bcl-2 expression in squamous cell carcinoma of the head and neck. Cancer 1999; 85:164 70
3. Oezzella F, Turley H, Kuzu I, et al. Bcl-2 protein in non-small cell lung carcinoma. N Engl J Med 1993;329.690 4.
4. Reeve JG, Xiong J, Morgean J, Blehen NM. Expression of apoptosis-regulatory genes in lung tumor cell lines: relationship to p53 expression and relevance to acquired drug resistance. J Cancer 1996;73: 193 200.
5. Reed JC, Miyashita T, Takayama S.et al. Bcl 2 family proteins: Regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 1996;60,23-32.
6. DiPaola RS, Aisner J. Overcoming Bcl 2 and p53 mediated resistance in prostate cancer. Semin Oncol 1999;26:112-6.
7. Adams JM, Cory S. Life or death decision by the bcl-2 protein family. Trends Biochem Sci 2001;26:61-6.
8. Antosson B, Martinou JC. The bcl 2 protein family. Exp Cell Res 2000; 256:50-7.
9. Antosson B. Bax and other pro-apoptotic bcl-2 family'killer-proteins' and their victim, the mitochondrion. Cell Tissue Res 2001;306:347-60.
10. Chi KC, Wallis AE, Lee CH.et al. Effects of Bcl 2 modulation with G3139 antisense oligonucleotide on human breast cancer cells are independent of inherent Bcl-2 protein expression. Breast Cancer Res Treat. 2000;63:199 212.
11. Lopes de Menezes DE, Hudon N, McIntosh N.et al. Molecular and pharmacokinetic properties associated with the therapeutics of Bcl 2 antisense oligonucleotide G3139 combined with free and liposomal doxorubicin. Clin Cancer Res 2000;6:2891-902.
12. Leech SH, Olie R A, Gautschi O, Simoes Wust P, Tschopp A, Haner S, Hall R, Stahel J, Zangemeister-Wittke RA. Induction of apoptosis in lung cancer cells following bcl xL anti sense treatment. Int J Cancer 2000;86:570-6.
13. Finnegan NM, Curtin JF, Prevost G, Morgan B, CotterTG. Induction of apoptosis in prostate carcinoma cells by BH3 peptides which inhibit Bak/Bcl 2 interactions Br J Cancer 2001;85:115-21.
14. Holinger EP, Chittenden T, Lutz RJ. Bak BH3 peptides antagonize Bcl-xL function and induce apoptosis through cytochrome c independent activation of caspases. J Biol Chem 1999,274,13298-304
15. Polster BM, Kinnally KW, Fiskum G. BH3 death domain peptide induces cell type-selective mitochondrial outer membrane permeability. J Biol Chem 2001,276 37887-94

16. Wang JL, Dongxiang L, Zhang Z-L, et al. Structure based discovery of an organic compound that binds Bcl 2 protein and induces apoptosis of tumor cells. Proc Natl Acad Sci USA 2000;97:7124-9.
17. Degterev A, Lugovskoy A,. Cardone M, Mulley B, Wagner G, Mitchison T, Juan J. Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-xL. Nature Cell Biol 2001;3:173-82
18. Enyedy IJ, Ling Y, Nacro K, et al. Discovery of small molecule inhibitors of Bcl-2 through structure based computer screening. J Med Chem 2001;44:4313-24
19. Feng WF, Liu FT, Patwari Y, Agrawal SG, Newland AC, Jia L. BH-3 domain mimetic compound BH3I-2' induces rapid damage to the inner mitochondrial membrane prior to the cytochrome c release from mitochondria Br J Haematol 2003; 21,332-40.
20. Stuschke M, Sak A, Wurm R, Sinn B, Wolf G, Stuben G, Budach V. Radiation induced apoptosis in human non-small-cell lung cancer cell lines is secondary to cell cycle progression beyond the G2 phase checkpoint. Int J Radiat Biol 2002;78: 807 9.
21 Streffer J, Rimner A, Rieger J, Naumann U, Rodemann HP, Weller M. Bcl-2 family proteins modulate radiosensitivity in human malignant glioma cells. J Neuro-Oncol 2002;56:43-9.
22. Kharbanda S, Pandey P, Schofield L, Israels S, Roncinske R, Yoshida K, Bharti A, Yuan ZM, Saxena S, Kufe D. Role of Bcl-xL as an inhibitor of cytosolic cytochrome c accumulation in DNA damage-induced apoptosis. Proc Natl Acad Sci USA 1997,94,6939-42
23 O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Kohn KW. Characterization of the p53 tumor suppressor pathway in cell lines of the national cancer institute anticancer drug screen and correlations with the growth inhibitory potency of 123 anticancer agents. Cancer Research 1997,57:4285 300.
24 Mitsudomi T, Steinberg SM, Nau MM, Carbone D, D'Amico D, Bodner S, Oie HK, Linnoila I, Mulshine JL, Minna JD, Gazdar AF. Oncogene 1992;7:171 80.
25 Roa WH, Chen H, Fulton D, Gulavita S, Shaw A, Th'ng J, Farr-Jones M, Moore R, Petruk K. X-linked inhibitor regulating TRAIL induced apoptosis in chemoresistant human primary glioblastoma cells. Clin Invest Med 2003; 26:231-42
26 Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB, Wild type p53 in a cell cycle checkpoint determinant following irradiation Proc Natl Acad Sci USA 1992;89: 7491-5.
27 Wu X, Levine A. p53 and E2F 1 cooperate to mediate apoptosis. Proc Natl Sci USA;91:3602-6.
28 Hao J, Ming Y, Feng-Ting L, Newland A, Jia, L. Bcl-2 Inhibitors Sensitize Tumor Necrosis Factor Related Apoptosis Inducing Ligand Induced Apoptosis by Uncoupling of Mitochondrial Respiration in Human Leukemic CEM Cells. Cancer Research 2004,63:3607-16.
29. Lowe SW, Schmitt EM, Smith SW, Osborne B, Jack T. Nature 1993;847-9
30. Haas-Kogan DA, Yount G, Haas M, Levi D, Kogan SS, Hu L, Vidair C, Deen DF, Dewey WC, Israel MA. P53-dependent G1 arrest and p53 independent apoptosis influence the radiobiologic response of glioblastoma. Int J Radiat Oncol Biol Phys 1996;36:95-103.
31. Brown JM, Wouters BG. Apoptosis,p53, and tumor cell sensitivity to anticancer agents Cancer Res 1999;59,1391-9.
32. Lee JM, Berstein A. P53 mutation increase resistance to ionizing radiation. Proc Natl Acad Sci USA 1993;90:5742-6.

Address correspondence to: Dr. Wilson Roa, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta, Canada, T6G 1Z2, Tel: (780) 432-8517, Fax: (780) 432-8380, e-mail: wilsonro@cancerboard.ab.ca